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TECHNOLOGICAL
UNIVERSITY**

PROTEIN KINASE C IN REGULATION OF EXOCYTOSIS

XUE RENHAO

**SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

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PKC IN REGULATION OF EXOCYTOSIS

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ENGINEERING**

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List of abbreviations

[Ca²⁺]_i	intracellular free Ca ²⁺ concentration
1-But	1-butanol
AM	acetoxymethyl
Fura-2-AM	fura-2 acetoxymethyl ester
ATP	adenosine tri-phosphate
BDM	2,3-butanedione 2-monoxime
BIS	bisindolylmaleimide
BoNT	botulinum neurotoxin
BoNT/E	botulinum neurotoxin E
BoNT/C1	botulinum neurotoxin C1
BSA	bovine serum albumin
CFM	carbon-fiber-microelectrode
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
dn	dominant negative
DNA	deoxyribonucleic acid
cDNA	complementary DNA
DsRed	Discosoma sp. Red
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
FBS	fetal bovine serum
GAP	growth-associated protein
GFP	green fluorescent protein
HCSP	highly Ca ²⁺ -sensitive pool
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HS	horse serum
IRES	internal ribosome entry site
LB	lysogeny broth
LCD	long coverage diameter

LDCV	large dense core vesicles
MARCKS	myristoylated alanine-rich protein kinase C substrate
MSD	mean square displacement
NPY	neuropeptide Y
NSF	<i>N</i> -ethylmaleimide-sensitive factor
PA	phosphatidic acid
PBS	phosphate buffer solution
PIP2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PMA	Phorbol-12-myristate-13-acetate
PSF	pre-spike foot
RNA	ribonucleic acid
mRNA	messenger RNA
siRNA	small interfering RNA
ROCK	Rho kinase
RRP	readily releasable pool
SAF	stand alone foot
SCD	short coverage diameter
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SNAP	synaptosomal-associated protein
SNAP-25	synaptosomal-associated protein of 25 kD
α-SNAP	alpha-synaptosomal-associated protein
SNARE	soluble <i>N</i> -ethylmaleimide-sensitive factor attachment protein receptor
v-SNARE	vesicle SNARE
t-SNARE	target-membrane SNARE
SRP	slowly releasable pool
TAE	tris-acetate- ethylenediaminetetraacetic acid

TIRF	total internal reflection fluorescence
TIRFM	total internal reflection fluorescence microscopy
VAMP	vesicle-associated membrane protein
wt	wild-type

Summary

Exocytosis, by which secretory vesicles are directed to outer cell membrane and subsequently discharge their contents upon triggering, is an important and ubiquitous cellular process responsible for secretion of neurotransmitters from neurons and hormones from endocrine cells. Exocytosis is highly regulated through the orchestrated actions of various proteins and lipids. It has been well recognized that protein kinase Cs (PKCs) are active regulators of exocytosis via phosphorylating multiple key proteins in the exocytotic cascade. The specific actions mediated by PKCs, however, have not been fully elucidated. The challenges mainly lie in the following aspects: 1) large variety of PKC isoforms and dubious specificity of the commonly used pharmacological tools; 2) large variety of PKC substrates; 4) difficulties to isolate the molecular steps in the highly dynamic exocytotic process.

In the present work, we studied PKC regulations at single cell level by combining electrochemical amperometry, bio-imaging, and molecular biology methods. Amperometry measurement based on carbon fiber microelectrode is able to directly detect exocytosis with single vesicle sensitivity and millisecond resolution without interference from endocytosis. It is particularly useful to reveal the detailed kinetics of quantal vesicle fusion catalyzed by secretory proteins such as SNAREs. Total internal reflection fluorescence microscopy (TIRFM) was used to visualize individual vesicles in the subplasmalemmal region and track their dynamic trafficking. Single-cell photometry

was used to measure intracellular Ca^{2+} concentration and dynamics. Finally, molecular engineering was used to construct plasmids of fluorescently labeled PKC isoforms.

We demonstrate that PKC modulates multiple aspects of exocytosis including vesicle vertical and lateral trafficking, the extent of exocytosis, vesicle fusion kinetics, and recovery of exocytosis after an exhausting stimulation. And PKC alpha and epsilon play distinct roles in those regulations.

CHAPTER 1

INTRODUCTION

1.1 Exocytosis is highly dynamic and highly regulated

Exocytosis, also known as regulated secretion, is the process by which secretory vesicles are directed to outer cell membrane and subsequently discharge their contents upon triggering (Zucker, 1996, Lin & Scheller, 2000, Burgoyne & Morgan, 2003). Exocytosis is an important and ubiquitous cellular process responsible for secretion of hormones from endocrine cells, release of neurotransmitters from presynaptic neurons, acrosome reaction during fertilization, delivery of plasma membrane bound receptors, etc (Sudhof, 1995, Henquin, 2000, Jahn & Sudhof, 1994, Wassarman, 1999, Rothman, 1994). In the past four decades or so, exocytosis has been a very actively pursued research area. Although the tremendous research efforts have been devoted to uncover the molecular mechanisms underlying exocytosis, the full picture of this complex process, which is highly regulated by a plethora of proteins and lipidic factors, has yet to be fully elucidated.

Exocytosis is a highly dynamic process involving a sequence of molecular steps including vesicle transport, tethering and docking onto plasma membrane, priming (or maturation), and final Ca^{2+} -dependent vesicle fusion (Fig. 1.1) (Sudhof, 2004, Becherer & Rettig, 2006, Izumi et al., 2007). After loaded with the secretory cargos such as

hormones, the vesicles are transported from the inner cytosol to the subplasmalemmal region along the microtubule and actin tracks by motor proteins (myosin II and V) (Trifaro et al., 2008, Loubery & Coudrier, 2008). The arrived secretory vesicles constantly explore and interact or tether with various secretory factors in the subplasmalemmal region. In a process termed as docking, the subplasmalemmal vesicles are brought into close contact with the plasma membrane when vesicle protein VAMP (vesicle-associated membrane protein) complexes with plasma membrane associating proteins SNAP-25 (synaptosomal-associated protein of 25 kD) and syntaxin. VAMP, SNAP-25 and syntaxin are collectively known as SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins (Sollner, 2003, Verhage & Sorensen, 2008). After docking on the fusion sites, the vesicles are primed (or matured) in an ATP (adenosine 5'-triphosphate) dependent manner to the readily releasable state (Chen et al., 2001). Finally, mediated by Ca^{2+} sensors (putatively, synaptotagmin), increase in the intracellular Ca^{2+} concentration triggers the final fusion between the vesicular membrane and the plasma membrane, likely driven by the twisting of the SNARE complex (Fig. 1.2) (Jahn et al., 2003).

Vesicle fusion starts with formation of a nanometric aqueous fusion pore through which secretory compounds escape from the vesicular lumen into extracellular space. Subsequently, fusion pore expands quickly to discharge the secretory compounds. The dilation of fusion pore may end with full collapse and complete mergence of vesicle membrane onto plasma membrane. Or it may terminate prematurely in a so called kiss-

and-run mode with only partial release of the contents (Jackson & Chapman, 2006, Burgoyne & Barclay, 2002).

Fig. 1.1 Synaptic membrane trafficking. (Gundelfinger E. D. et. al. 2003. *Nature Reviews Molecular Cell Biology* 4: 127-139)

Fig. 1.2 Vesicle docking, priming and fusion. (Chapman E. R. 2002. *Nature Reviews Molecular Cell Biology* 3: 498-508)

A plethora of secretory proteins play important regulatory roles at distinct stages of exocytosis, through spatially and temporally coordinated protein-protein and protein-lipid interactions (Burgoyne & Morgan, 2003). Among these proteins, the SNAREs are the core engine of the exocytotic fusion. Since synaptic SNAREs were first purified in 1993 (Sollner et al., 1993b), several lines of evidence has demonstrated their crucial functions in regulated secretion of yeast (Gerst, 1999), drosophila (Schulze et al., 1995) and mouse (Schoch et al., 2001). The vesicle SNARE protein (v-SNARE), VAMP, and two target-membrane SNARE proteins (t-SNARE), SNAP-25 and syntaxin, form a stable protein complex upon vesicle docking (Sollner et al., 1993a). In the SNARE complex, the H3 helix of syntaxin interacts with the VAMP coiled-coil domain and two SNAP-25 helices to form coiled-coil bundles. This process proceeds down the energy gradient and brings the lipid bilayers of the vesicle membrane and plasma membrane sufficiently close to overcome the hydration barrier and initiate membrane fusion (Burgoyne & Morgan, 2003).

A number of regulatory proteins in the exocytotic pathways have been shown to play their roles via interactions with SNARE proteins. For example, α -SNAP (alpha-synaptosomal-associated protein) and NSF (*N*-ethylmaleimide-sensitive factor) are known as molecular chaperones in charge of the ATP-dependent disassembly of SNARE complex (Hayashi et al., 1995, Ungermann et al., 1998). Munc18 regulates vesicle docking and priming via binding with syntaxin (Voets et al., 2001, Rizo & Sudhof, 2002) whereby the formation of SNARE complex is inhibited (Dulubova et al., 1999). Synaptotagmin, which serves as the Ca^{2+} sensor of final fusion (Brose et al., 1992, Geppert et al., 1994), has been reported to bind with SNARE complex as a clamp of

fusion before accessed by Ca^{2+} (Chapman et al., 1995, Schiavo et al., 1996). Munc13 which has been show to regulate vesicle priming (Ashery et al., 2000), also has a critical interaction with syntaxin (Betz et al., 1997). Besides proteins, various lipids also participate in the regulation of exocytosis. A typical example is phosphatidylinositol-4,5-bisphosphate (PIP₂), which is likely to mediate vesicle release via recruiting diverse protein factors to the fusion site (James et al., 2008).

In addition to protein-to-protein or protein-to-lipid interaction initiated regulations, exocytosis is acutely modulated by protein phosphorylation mediated by various protein kinases such as protein kinase A (PKA) (Seino & Shibasaki, 2005), Rho kinase (ROCK) (Mueller et al., 2005), and most notably, protein kinase C (PKC).

1.2 Protein kinase C and its regulatory roles in exocytosis

1.2.1 Protein kinase C family

PKC is a family of protein kinases that phosphorylate their protein substrates on serine and threonine residues (Newton, 1995). It is well recognized that PKC participates in various physiological processes including cell growth and differentiation, tumor promotion, nerve system development, smooth muscle cell contraction, oxidative injury, aging, apoptosis and exocytosis (Clemens et al., 1992, Nishizuka, 1986, Liu, 1996, Gopalakrishna & Jaken, 2000, Brodie & Blumberg, 2003).

The family of PKC consists of at least ten members, including conventional PKCs (α , β , and γ) whose activation requires both Ca^{2+} and diacylglycerol (DAG), novel PKCs (δ , ϵ , η , and θ) whose activation only relies on DAG, and atypical PKCs (ζ , τ , λ) which are activated by neither Ca^{2+} nor DAG (Kikkawa et al., 1989, Hug & Sarre, 1993, Morgan et al., 2005). PKC is a single polypeptide chain and the primary amino acid sequence of PKCs is made up of four highly conserved domains: C1, C2, C3 and C4 (Azzi et al., 1992, Liu, 1996), among which C3 and C4 make up the kinase core at C-terminal and C1 and C2 are N-terminal regulatory domains (Fig. 1.3) (Parker et al., 1986, Newton, 2003). The C1 domain is responsible for the binding of DAG while the C2 domain binds anionic phospholipids in a Ca^{2+} -dependent manner due to the presence of several invariant Ca^{2+} -binding residues in the structure (Lee & Bell, 1986). The conventional PKC isoforms contain both of the two regulatory motifs (C1 and C2). Similarly, the novel PKC isoforms have twin C1 domains and a C2 domain-like sequence (novel C2) in their N-terminal regulatory regions. However, unlike the conventional PKCs, the novel C2 sequence lacks side chains bearing Ca^{2+} -coordinating acidic residues. Hence, novel PKCs can be maximally activated by DAG in the absence of Ca^{2+} . The atypical PKC isoforms lack the Ca^{2+} -sensitive C2 domain and contain only a single cysteine-rich zinc finger structure (atypical C1 domain) which is insensitive to DAG binding. As a result, neither Ca^{2+} nor DAG regulates the atypical PKC isoforms (Coussens et al., 1986). The kinase core of PKCs includes an acidic amino acid sequence in the C4 region which serves as a substrate-recognizing structure while an ATP binding site within a 40-residue amino acid sequence presents in the C3 section (Hanks et al., 1988). When the kinase core binds to both ATP and substrate, the phosphorylation of the substrate is catalyzed.

Fig. 1.3 Primary structure of PKC isoforms. (Newton A. C. 2003. *Biochemical Journal* **370**: 361-371)

Phorbol esters such as phorbol-12-myristate-13-acetate (PMA) are DAG analogues and have been widely used as a PKC activator as it binds to the C1 domain of conventional and novel PKCs (Bell & Burns, 1991, Nelsestuen & Bazzi, 1991). On the other hand, bisindolylmaleimides (BIS) that block the ATP binding sites in the C3 catalytic domain of PKCs often serve as PKC inhibitors (Toullec et al., 1991). Using these pharmacological tools, it has been revealed, by biochemical (Ozawa et al., 1993, Soliakov & Wonnacott, 2001), electrophysiological (Borges et al., 2008, Chen & Gillis, 2000, Heinemann et al., 1994) and bio-imaging (Holz & Axelrod, 2008, Steyer et al., 1997) approaches, that PKC is playing a versatile role in regulating exocytosis. In fact, several key secretory proteins, such as SNAP-25 (Shimazaki et al., 1996, Nagy et al., 2002), synaptotagmin (Hilfiker et al., 1999, Haberman et al., 2005), Munc18 (Fujita et al., 1996, Barclay et al., 2003, Nili et al., 2006), and NSF (Matveeva et al., 2001) have been recognized as PKC substrates.

1.2.2 PKC mediates F-actin rearrangement and enhances vesicle recruitment to the subplasmalemmal region

Imaging experiments by transmission electron microscope and confocal microscope have suggested that activation of PKC may enlarge the extent of exocytosis through increasing the vesicle number close to the plasma membrane in bovine chromaffin cells (Vitale et al., 1995), neuroblastoma SH-SY5Y cells (Danks et al., 1999) and PC12 cells (Shoji-Kasai et al., 2002). It has also been demonstrated that following activation of PKC, the transport of secretory vesicles to the plasma membrane is facilitated, possibly by partial disassembly of cell cortical F-actin cytoskeleton (Eitzen, 2003). Cells possess a dense actin meshwork underneath the plasma membrane. Agents that depolymerize actin filaments potentiate exocytosis at low doses but progressively impair it at higher concentrations as reported in PC12 (Matter et al., 1989) and chromaffin cells (Gasman et al., 2004). Based on these results, it is assumed that cortical F-actin network may play a dual role in membrane recruitment of vesicles. On one hand, the cortical F-actin network functions as a physical barrier separating a large pool of the reserved vesicles in the cytosol and the docked vesicles on the plasma membrane. It needs to be temporally removed to allow reserved vesicles to undergo tethering, docking and further exocytotic processes. On the other hand, once docked on the plasma membrane, F-actin may serve as an active regulator to keep the vesicles in place and provide driving force for the final membrane fusion (Eitzen, 2003).

Several lines of evidences have been documented that PKC activation (e.g., by PMA) causes disassembly and reorganization of dense actin cortex clearing the way for myosin-dependent transportation of vesicles from the reserve pool to the plasma membrane along the remaining fine actin filament (Trifaro et al., 2002). PKC activation disrupts and reorganizes actin networks probably through multiple pathways, including recruitment of actin severing protein scinderin (Eitzen, 2003) and phosphorylation of MARCKS (myristoylated alanine-rich protein kinase C substrate) (Tapp et al., 2005, Trifaro et al., 2002) or other actin-filament-associated proteins such as adducin and fascin (Larsson, 2006). Additionally, annexins I and II, growth-associated protein (Moisenovich et al.) (Gil et al., 2000) and Rho family GTPase such as RhoA and Cdc42 (Ridley, 2006) might be involved in the PKC-actin pathways as well. In addition, the motor proteins (myosin), which serve as the vehicles of vesicle trafficking, might be a target of PKC as well (Naka et al., 1983, Ludowyke et al., 1989). Due to the complexity of the PKC centered pathway network, the mechanisms of PKC-mediated recruitment of vesicles to subplasmalemmal region, however, still remains debated and needs further studies.

1.2.3 PKC phosphorylates SNAP-25 and Munc18 and modulates readily releasable pool (RRP)

It is commonly believed that readily releasable pool (RRP) exists in secretory cells, which consists of vesicles that can be released immediately upon stimulation without further maturation steps (Sorensen, 2004). This RRP was found in endocrine cells such as bovine chromaffin cells using capacitance measurements based on patch clamp technique

(Horrigan & Bookman, 1994, Gillis & Chow, 1997). Following photolysis of caged Ca^{2+} which rapidly elevates the intracellular calcium concentration, there are two components of the exocytotic burst: a fast phase of a time scale of tens of milliseconds followed by a slower and sustain phase. The fast phase may represent secretion of those fully matured vesicles from RRP. The slower phase is thought as secretion of vesicles from the slowly releasable pool (SRP) which refills the depleted RRP through further maturation steps (Sorensen, 2004).

Studies based on activation of PKC by phorbol esters have shown enlargement of RRP size in chromaffin cells (Gillis et al., 1996) and in hippocampal neurons (Stevens & Sullivan, 1998). It has also been suggested in chromaffin cells that PKC activation increases not only the size of RRP but also the rate of replenishment from SRP (Smith et al., 1998).

Further studies revealed that the C terminus of SNAP-25 participates in vesicle recruitment, which is regulated by PKC-dependent phosphorylation (Nagy et al., 2002). Moreover, Munc18-1, which attenuates secretion by binding to syntaxin-1a and consequently preventing SNARE complexes formation, can be phosphorylated by PKC. And such phosphorylation may discourage the formation of Munc18-1-syntaxin-1a complexes, thus facilitate SNARE complex forming and potentiate vesicle pool replenishment (Nili et al., 2006). These findings postulate the roles of PKC in modulating of RRP via regulating the vesicle docking and priming processes. Nonetheless, as the

molecular identities of RRP are not fully understood, the mechanisms of PKC regulations on RRP are still largely unknown.

1.2.4 PKC phosphorylates synaptotagmin and increases Ca^{2+} sensitivity of vesicle release

Recently, it was reported that PKC enhances exocytosis by directly shifting vesicles to a highly Ca^{2+} sensitive state in gonadotropes (Zhu et al., 2002), pancreatic β cells (Wan et al., 2004) and chromaffin cells (Yang et al., 2002). Yang et al. suggested the existence of a small “highly Ca^{2+} -sensitive pool” (HCSP) in the Ca^{2+} dependent exocytosis at low intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) levels in chromaffin cells (Yang et al., 2002). Further studies showed that the size of HCSP is enlarged by phorbol ester, whereas the rate constant is not affected (Yang & Gillis, 2004). These observations are attributed to the effects of PKC to transform readily releasable vesicles further into a state of higher Ca^{2+} -sensitivity. However, the underlining mechanism is unknown. Synaptotagmin, the putative Ca^{2+} sensor for exocytosis, is an attractive candidate as it is an in vitro PKC substrate and has also been shown to be phosphorylated in cells (Haberman et al., 2005, Hilfiker et al., 1999).

1.2.5 PKC regulates the fusion pore opening kinetics

Quantal analysis based on carbon-fiber-microelectrode (CFM) amperometric recording has shown that activation of PKC alters the kinetics of single vesicle fusion events. A

reduction in quantal size was observed in some (Vanderkloot, 1991) but not all synapses (Yawo, 1999) after PKC activation. Attenuation in quantal size accompanied with increase of the rate of fusion pore dilation was also found in chromaffin cells, suggesting that PKC activation likely leads to a switch towards fast kiss-and-run process (Graham et al., 2000, Borges et al., 2002). In this process, vesicles partially release their contents through a transiently opened fusion pore (Harata et al., 2006).

PKC phosphorylates the fusion machineries such as SNAP-25 (Nagy et al., 2002). However, botulinum neurotoxin E (BoNT/E) which cleaves SNAP-25 and botulinum neurotoxin C1 (BoNT/C1) that cleaves syntaxin only inhibit the extent of exocytosis, but do not affect quantal fusion kinetics (Graham et al., 2002). These findings may lead to a speculation that the SNARE complex actually does not modulate the kinetics and quantal size of fusion despite its essential role in driving the final fusion. Barclay et al. suggested that phosphorylation of Munc18 is likely to be involved in the changes of exocytosis kinetics in response to phorbol ester (Barclay et al., 2003). PKC phosphorylates Munc18 at Ser313 and Ser306 (Craig et al., 2003, Barclay et al., 2003). Overexpression of R39C mutation of Munc18, which loses the capability to bind syntaxin, leads to faster initial release rate of catecholamines from the large dense core vesicles (LDCV) followed by abrupt termination (as the consequence, amperometric signals with similar amplitude and decreased quantal charge were observed), similar to that in the PMA pretreated cells (Fisher et al., 2001). But, in PC12 cells, a similar R39C mutation of Munc18 failed to alter the quantal size and kinetics of fusion events (Schutz et al., 2005). Such contradictory observations demand further investigations. Additionally, the Ca^{2+} sensor

of final fusion (synaptotagmin) which has been shown to regulate fusion pore dynamics (Wang et al., 2001) is a substrate of PKC as well (Hilfiker et al., 1999, Haberman et al., 2005).

In summary, PKC is an essential regulator of exocytosis. It plays multiple roles in different aspects of exocytosis process. The functions of PKC are likely through mediating the phosphorylation of various substrates. However, the underlying mechanisms and isoform specificity are still far from fully understood, because of the diverse roles of PKC, large variety of PKC isoform and limitations of the pharmacological tools and traditional assay techniques. We aim to further explore the regulation mechanisms of PKC by combining methods in molecular biology, bioimaging, and single cell recordings.

1.3 Total internal reflection fluorescence (TIRF) imaging and tracking of vesicle motion

A complicated pattern of vesicle trafficking is found in the subplasmalemmal region of synapses (Zenisek et al., 2000) and secretory cells (Johns et al., 2001). To visualize the transport phenomena of vesicles prior to their release can provide insights into the transition of vesicle between different molecular states. Such direct visualization became possible with the recent advent of total internal reflection fluorescent microscopy (TIRFM), which enables real-time imaging of individual fluorescent-tagged secretory vesicles near the plasma membrane. Because the evanescent field of TIRFM selectively

illuminates the thin section (~200 nm) just above the interface between the glass coverslip and the adhered cell, TIRFM is instrumental to gain insights into dynamic events occurring at, or close to, the plasma membrane of living cells with outstanding optical contrast and resolution.

Fig. 1.4 Optical pathways of total internal reflection fluorescence microscopy (TIRFM). (Olympus co. ltd. <http://www.olympusamerica.com>)

Total internal reflection fluorescence (TIRF) is an optical technique, developed in early 1980s in order to study the molecular phenomena at the liquid-to-solid interfaces, especially well-suited for visualization of the regions underneath plasma membrane at cell-substrate contacts (Axelrod et al., 1983). As shown in Fig. 1.4, at the interface of glass (high refraction index) and aqueous solution (low refraction index), an incoming light beam will undergo total internal reflection if the incident angle θ (the angle of the incident light beam measured from the normal of the interface) is larger than the “critical

angle” θ_c , which is estimated from Equation 1.1 (n_1 and n_2 represent the refraction index of the solid and liquid phases, respectively).

$$\theta_c = \sin^{-1}\left(\frac{n_2}{n_1}\right) \quad \text{Equation 1.1}$$

The total internal reflection results in what is often called an “evanescent field”, which is a field that penetrates a tiny distance into the liquid phase and decays exponentially with the penetrating distance, illuminating the fluorophores in this thin layer critically beneath the interface. The characteristic penetrating distance depends on the incident angle θ , which is only thirty to a few hundred nanometers. The penetrating distance of evanescent wave corresponds to the thickness of TIRF sampling section (Schmoranzner et al., 2000) (Fig. 1.4). Only fluorescent molecules within this sampling section can be excited. As a result, the influence of out-of-focus fluorescent signal is largely attenuated, enabling high signal-to-background imaging of interface events (Steyer et al., 1997). Furthermore, the evanescent wave occurs transiently in response to the incident laser beam, so that the fluorescent molecules in the evanescent field can be excited instantly without any scanning process as is required in laser scanning microscopy. This makes it possible for real time imaging with extremely high time resolution. In the meantime, since transient illumination instead of scanning is applied, the exposure time of fluorescent molecules to the excitation can be minimized, resulting in minimal photo-bleaching, and photo-toxicity.

The advantages of low background, high time resolution, minor photo-bleaching, and photo-toxicity have made TIRFM an excellent technique for visualization of the position,

motion dynamics, concentrations, and reactions of fluorophores close to the interface. For cellular biology, the method can be utilized to observe molecular processes, even single molecular interactions, occurring at or just beneath the plasma membrane. As applied to studies on exocytosis, TIRFM is a powerful tool to reveal the dynamics of single secretory granules before release as well as the release of dye following exocytosis (Oheim et al., 1998a). Direct TIRFM visualization of fluorescence-labeled LDCVs in living cells was first demonstrated in 1998 (Oheim et al., 1998b). This novel method constitutes a novel assay for exocytotic process in addition to biochemical, electrophysiological and electrochemical measurements.

However, TIRFM cannot image the vesicle residing deeply in the inner cytosol as the maximal thickness of the evanescent field is limited to hundreds of nanometers (Li et al., 2004). What's more, although TIRFM offers higher time resolution comparing with confocal imaging, its temporal resolution cannot reach millisecond scale. Therefore, it is very difficult to capture the details of the vesicle fusion events which take less than ten milliseconds as revealed by the amperometry technique.

1.4 Amperometry measurement using carbon fiber microelectrode (CFM)

Amperometric recording using CFM is an advanced technique allowing direct detection of exocytosis with single vesicle resolution without interference by endocytosis. It is particularly useful to reveal, with millisecond resolution, the details of vesicle fusion kinetics catalyzed by a number of fusion proteins, perhaps most importantly SNARE

proteins which are widely believed as the essential molecular machinery to enable membrane fusion (Jahn & Scheller, 2006).

CFM amperometric detection of secretion was first demonstrated by Wightman et al. in 1991 (Wightman et al., 1991), taking advantage of the fact that some secretory compounds, such as the indoleamines (e.g. 5-hydroxytryptamine) and the catecholamines (dopamine, epinephrine, and norepinephrine), can be readily oxidized on the voltge-biased electrode producing measurable transient current signals. Each amperometric current spike corresponds to an event of single vesicle release. And it provides information on the quantity of molecules oxidized at the tip of the microelectrode (Travis & Wightman, 1998) and the kinetic details of vesicle fusion process with millisecond temporal resolution.

The microelectrode is usually fabricated from a carbon fiber with a diameter $< 40\text{ }\mu\text{m}$. The carbon fiber is coated by insulating material to reduce capacitance induced noise except the exposed tip as the sensing region. Smaller electrode diameter gives a smaller double layer capacitance ($\sim 40\text{ }\mu\text{F}/\text{cm}^2$) therefore less recording noise (Howell et al., 1986). On the other hand, too small a tip can only record a small fraction of exocytosis from the entire cell membrane. CFM with $5\text{ }\mu\text{m}$ diameter is used in our experiments,

Biasing the CFM at a constant potential, the exocytotic events can be recorded as amperometric spikes— electrochemical current resulted from monoamine oxidation induced electron transferring. The total charge (Q , the time integral of the current) in

coulombs is proportional to the quantity of detected molecules (N) by Faraday's law (Equation 1.2) where n is the charge transferred from one single monoamine molecule in the electrochemical reaction ($n = 2$ for catecholamines) and F is Faraday's constant (96,485 C/mole).

$$Q = nFN \quad \text{Equation 1.2}$$

So, the quantal size, or the released number of neurotransmitters per vesicle, can be estimated by calculating the integral of each current signal. The number of recorded spikes directly indicates the number of secreted vesicles, while the shape of the amperometric signal reflects the dynamics of the quantal vesicle fusion process. As shown in Fig. 1.5, the foot signal preceding the amperometric spike (PSF) reveals the initial opening of the fusion pore, as it arises from the slow leakage of the vesicular content through initial fusion pore formed between the vesicular and the plasma membranes (Fig. 1.6C). The rising phase of the spike represents the subsequent expansion of the fusion pore. The rise time (t_{rise}) and rise slope can be analyzed as the indications for the expansion time and expansion rate of the fusion pore respectively (Fig. 1.6B). Fusion pore may close prematurely without discharging all the vesicle content inside giving rise to a kiss-and-run type of amperometric event often evidenced by a fast decay, small quantal size, or even stand alone foot signal (SAF). Alternatively, it may dilate fully to expel the entire vesicle content giving rise to a relatively larger quantal signal. The half width of the spike, also called $t_{1/2}$, (Fig. 1.6A) is another important kinetics parameter representing the total time of the fusion process. Statistical analysis of

these spike parameters can provide detailed information in fusion pore dynamics, from which the actions of the molecular machineries in charge of membrane fusion, such as SNARE proteins, can be inferred (Mosharov & Sulzer, 2005).

Fig. 1.5 The amperometric spike and the related single vesicle fusion kinetics. (Evanko D. 2005. *Nature Methods* 2: 650-650)

Fig. 1.6 Parameters of amperometric spikes. **A.** Spike peak amplitude (I_{max}), half width ($t_{1/2}$) and total charge (Q); **B.** Spike rise time (t_{rise}) and rise slope; **C.** Charge (Q_{foot}), amplitude (I_{foot}) and time duration (t_{foot}) of prespike foot (PSF). (Mosharov E. V. & Sulzer D. 2005. *Nature Methods* 2: 651-658)

Exocytotic events occurring further away from the tip of the carbon fiber are detected with diffusional delays and attenuated to variable degrees. In such a case, a portion of released molecules will diffuse away and never hit the electrode surface, and the resulting

amperometric signal is distorted due to the slow diffusion process. Therefore, CFM is usually positioned very close to or on the cell surface. As a result, only ~5% of the release events occurring on the cell membrane can be detected due to the small size of CFM and its planar geometry. Although amperometry is an extraordinary tool to examine the quantal fusion event, it is not able to provide direct information on the early steps of exocytosis.

1.5 Research Motivations and Objectives

Exocytosis is a biological phenomenon of great physiological significance. Its malfunctions lead to neurodegenerative diseases, hormone disorders and other critical diseases. For example, Parkinson's disease is caused in part by secretion deficiency of neurotransmitters in the central nervous system. As another example, diabetes type 2 is partly due to deficiency in insulin secretion from pancreatic beta cells. About 9% and 0.4% of the adult population in Singapore are suffering from diabetes and Parkinson's disease respectively. There is still no cure for these diseases. Current medications or surgery can only provide relieves from the symptoms. Our studies provide new insights to the mechanisms of regulated secretion in nervous system and endocrine system, and therefore may provide clues for better treatments or cures to these related diseases in the future. We focus on the PKC which plays a central role in regulating exocytosis via interactions with other secretory factors. Chemicals targeting on the PKC regulatory pathways may be potential drugs to fight with secretion-related diseases. But to decipher the complex PKC regulations is very challenging because of the diversity of PKC

isoforms and their substrates, and due to the limitations of the methods conventionally used to study the problem. We aim to study PKC regulations and its isoform specificities using unique single cell study platform based on the approaches of advanced bioimaging, electrochemical detection, and molecular biology.

1.5.1 Setting up the experimental platform for single cell assays to study the exocytotic process

Conventionally, exocytosis is studied using biochemical assays. However, such conventional measurements can only provide ensemble measurements from a population of cells with low temporal resolution (minutes to hours). Clearly, such assays are not able to resolve the dynamic exocytotic steps which can occur in seconds or even millisecond timescale and the detailed exocytotic kinetics from individual cells.

We will investigate exocytosis at single cell level by combining molecular biology, bioimaging, and electrochemical approaches. Firstly, we used molecular engineering to construct fluorescent protein conjugated PKC (or its mutants) plasmids. Overexpression of specific PKC isoforms or their mutants is a better way to selectively assess their function comparing to widely-used pharmacological manipulations which non-selectively inhibit or stimulate activities of multiple PKC isoforms and produce non-specific effects independent of PKC pathways. The co-expression of the fluorescent reporter protein allows us to identify and selectively record the cells in which the introduced PKC proteins are abundantly expressed. Secondly, we applied TIRFM imaging to track single

vesicle motion and fusion. LDCVs in PC12 cells are fluorescently labeled by overexpressing enhanced green fluorescent protein (EGFP) conjugated neuropeptide Y (NPY). In addition, an analytical framework has been developed to quantitatively assess vesicle trafficking. Thirdly, we employed amperometry measurement based on CFM to examine the kinetics of single vesicle fusion with millisecond resolution. Exocytosis is highly Ca^{2+} -dependent. Increase in the intracellular Ca^{2+} concentration triggers the final vesicle fusion and facilitates vesicle docking and priming. We used single-cell photometry to report the concentration and dynamics of intracellular Ca^{2+} .

In contrast to conventional ensemble biochemical assays, our single-cell platform will allow us to examine specific molecular actions along the secretion pathway with high sensitivity and temporal resolution.

1.5.2 Study the specific effects of PKC on different aspects of exocytosis and reveal differential actions of PKC isoforms

Although the regulatory roles of PKC have been well recognized, how a specific aspect of exocytosis is regulated by a specific PKC isoform is poorly elucidated, partly due to the diversity of PKC isoforms, the dubious specificity of the commonly used pharmacological agents, and the difficulties to resolve the molecular steps in the dynamic exocytotic cascade. PKC family consists of at least ten members which has different activation mechanism, phosphorylation targets, and spatial distribution. Presumably, different isoforms play distinct roles in exocytosis. Most studies of PKC functions rely on

the pharmacological tools. But these pharmacological agents such as PMA and BIS activate or inhibit multiple PKC isoforms nonselectively. Moreover, these activators and inhibitors may even exert nonspecific effects irrelevant to PKC pathways. For example, other DAG/PMA receptors, such as Munc13, also implicate in the exocytosis process (Rhee et al., 2002). And it has been demonstrated that BIS may impair exocytosis through nonspecific actions (Alessi, 1997).

Recently, studies have been carried out to identify the distinct roles of specific PKC isoforms. It was shown that alpha isoform of PKC is involved in vesicle trafficking in neuroblastoma SH-SY5Y cell (Danks et al., 1999) and docking in endothelial cell (Fu et al., 2005). The association of PKC ϵ (but not alpha) with secretory vesicles was demonstrated to be important to insulin secretion in beta cells (Mendez et al., 2003). And PKC ϵ was found to be critical in activity-dependent potentiation of LDCV exocytosis in bovine chromaffin cell (Park et al., 2006) and phorbol ester induced synaptic potentiation in rat nerve terminal (Saitoh et al., 2001). In pancreatic beta cells, PKC δ was reported to play a role in post-docking step (Uchida et al., 2007). And in rat chromaffin cells, PKC θ was shown to regulate the quantal size of vesicle fusion (Staal et al., 2008).

Using our single-cell platform and neuroendocrine PC12 cells as model, we examined the PKC effects on vesicle lateral and vertical trafficking, extent and rate of exocytosis, kinetics of quantal vesicle fusion. Among all the PKC isoforms, we focus particularly on alpha and epsilon isoforms, because PKC ϵ is the most abundant isoform and PKC α is the most constitutively active PKC isoform in rat chromaffin cell derived PC12 cells

(Wooten *et al.*, 1992). To avoid the caveats of the pharmacological manipulations, our study relies on overexpression of the wild-type or dominant negative forms of these PKC isoforms. Such studies shall add new dimensions to our understanding of cell secretion, this fundamental, ubiquitous and complicated physiological process.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and Solutions

The bath solution contains (in mM): 140 NaCl, 5.5 KCl, 2 MgCl₂, 2 CaCl₂, 10 glucose, and 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (titrated to pH 7.4). High K⁺ solution used to induce exocytosis in PC12 cells contains (in mM): 37 NaCl, 105 KCl, 6 CaCl₂, 2 MgCl₂, and 10 HEPES (titrated to pH 7.4). For PMA (phorbol-12-myristate-13-acetate) treatment, cells were incubated in the bath solution with addition of 0.1 μM PMA for 10 min. In some experiments, 2.5 μM BIS (bisindoylmaleimidine) was applied to the bath solution for 30 min before the introduction of PMA. For neomycin pretreatment, cells were incubated in the bath solution with addition of 100 μM neomycin for 30 minutes. All chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2 Cell Culture

PC12 is a pheochromocytoma cell line from rat (*Rattus norvegicus*), with the ability to produce and secrete catecholamine, including dopamine, epinephrine and norepinephrine, which is a good model for neuroendocrine cell secretion study (Greene & Tischler, 1976, Westerink & Ewing, 2008). Our PC12 cell line was purchased from American Type

Culture Collection (Rockville, MD, USA). All the culture medium and supplements are from Invitrogen (Rockville, MD, USA).

PC12 cells in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 5% heat-inactivated horse serum (HS) and 1% penicillin-streptomycin are maintained in a water jacketed CO₂ incubator (NuAire Inc., Plymouth, MN, US) with a humidified atmosphere of 5% CO₂ / 95% air at 37°C. The growth medium is renewed every 1 to 2 days. Cells are subcultured every 4-5 days using a trypsin free protocol. Briefly, the culture medium was removed from the flask with a sterilized serological pipette. Then the cells were detached by gentle rinsing with a 1 ml pipette tip and subsequently planted in new flasks or dishes at a split ratio of 1:4.

For long-term storage of the cells, the detached cells were resuspended in freezing medium, which contains 90% of growth medium and 10% of dimethyl sulfoxide (DMSO). The cell suspension was then transferred to the freezing vials, and stored in a freezing container at -80°C. In the container, the temperature slowly decreases at a rate of about 1°C/min. After overnight in the freezing container, the vials were moved into liquid nitrogen (about -196°C) for long time storage. To recover the cells from the freezing stock, the vials were transferred from liquid nitrogen to a 37°C water bath as quickly as possible. Right after thawed, the cell suspension was transferred to centrifuge tubes with warm culture medium, and centrifuged at 1200 rpm for 5 min. Then the cells were resuspended and washed again with fresh culture medium to completely remove the

DMSO. After washing, the cells were planted in flasks or dishes and maintained in normal culture conditions as described above.

Before experiment, PC12 cells were pretreated with 5 μ M dexamethasone for 3-5 days in normal culture condition as previous described (Tischler et al., 1983, Westerink & Ewing, 2008) to enhance chromaffin cell phenotype. Subsequently, PC12 cells were planted on 0.1 mg/ml poly-L-lysine pre-coated coverslips 1~3 days prior to experiments.

2.3 The setup for single cell recording

EPC-10 double patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) (Fig. 2.1B), which is of extremely low noise and high sampling frequency, is the state-of-the-art amplifier for single cell recording. All settings of the amplifier are under computer control and the instrument has built-in digital to analog converters and analog to digital converters. The amplifier and data acquisition are controlled by Patchmaster (HEKA) software.

To conduct measurements on single living cells, one must be able to view cells, manipulate the electrode onto a cell and avoid the inference from mechanical vibration and electrical noises. The microscope and the recoding platform are placed on an anti-vibration table (Technical Manufacturing Corporation, Peabody, MA, USA) (Fig. 2.1C), and enclosed in a Faraday cage to shield electrical interference from the environment (Fig. 2.1D) (Penner, 1995).

Fig. 2.1 The setup of single cell recording. **A.** An overview of the single cell recording work station. **B.** EPC-10 double patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany). **C.** Anti-vibration table (Technical Manufacturing Corporation, Peabody, MA, USA). **D.** Faraday cage (HEKA). **E.** Carbon fiber microelectrode (ALA Scientific Instruments, Westbury, NY, USA). **F.** PCS-5000 micromanipulator (Burleigh, Germany). **G.** IX71 inverted microscope (Olympus, Japan). **H.** The headstage of the HEKA EPC-10 amplifier.

The electrode (Fig. 2.1E) is mounted on a PCS-5000 piezoelectric micromanipulator (Burleigh, Germany) (Fig. 2.1F) in order to be positioned onto the target cell with fine control (60 nm precision) under an IX71 inverted microscope (Olympus, Japan) (Fig. 2.1G), and connected to the headstage of the HEKA amplifier (Fig. 2.1H) for pre-amplification.

The inverted fluorescence microscope can be used to locate the cells that express fluorescent report proteins (e.g. EGFP). And it is equipped with a photometry system (Fig. 2.5) for Ca^{2+} imaging at single cell level. In summary, our single-cell recording setup is able to recording electrical signals and optical signals from single cells with high sensitivity and high temporal resolution.

2.4 Amperometry measurement

2.4.1 Experimental protocols

Before experiments, PC12 cells were planted on poly-L-lysine pre-coated coverslips and transfected with certain plasmid or treated with certain drug when needed. All the amperometry recordings were performed at room temperature.

Exocytosis was induced by perfusing the high K^+ solution through a glass application micropipette (tip size of 2~3 μm) positioned 5~10 μm away from the cell by the Burleigh

manipulator. The application micropipettes were pulled from B100-50-10 borosilicate glass capillaries (O.D.: 1.0 mm, I.D.: 0.50 mm, length: 10 cm) (Sutter Instrument Co., Novato, CA, USA), using a P-2000 Laser Based Micropipette Puller (Sutter).

The carbon-fiber-microelectrodes (CFMs) (Fig. 2.1E) were purchased from ALA Scientific Instruments (Westbury, NY, USA). The microelectrodes are 5 μm in diameter and insulated with electrically deposited, highly inert paint. The carbon fiber tip was cut to expose a fresh surface before every recording in order to assure consistent sensitivity thus fidelity in reporting the true amperometric kinetics. The CFM, which is mounted on the micromanipulator and electrically connected to the amplifier, was gently positioned onto the cell membrane to avoid diffusion caused distortion in the signals (Fig. 2.2). The microelectrode was biased at 700 mV and the amperometric signals were sampled at 4 kHz and filtered at 1 kHz.

Fig. 2.2 Amperometry recording of PC12 cells. The CFM (right) is positioned onto the cell membrane and the application micropipette (left) is positioned 5~10 μm away from the cell. The inset shows an amperometric spike corresponding to a single release event of a secretory vesicle.

2.4.2 Data Analysis

All the data analyses were performed using Igor pro 6.0.1 (WaveMetrics, Lake Oswego, OR, USA). Only spikes with amplitudes larger than 2 pA (5 times of the background noise) were considered as true amperometric events. The parameters of amperometric spikes ($I_{\max}$, Q , $t_{1/2}$, t_{rise} and rise slope) and pre-spike foot (I_{foot} , t_{foot} , Q_{foot}) were obtained using an Igor program, Amperometric Spike Analysis 8.15, developed by Dr. Eugene Mosharov (Department of Neurology, Columbia University) (Mosharov & Sulzer, 2005).

Briefly, the spike amplitude ($I_{\max}$) is defined as the current amplitude from the peak to the baseline of the amperometric spike. The quantal size of vesicle release, or the total charge (Q) is obtained from integration of the amperometric current. The half width of the spike also called $t_{1/2}$ is the time duration estimated at 50% of the maximal amplitude between spike rising and falling. The parameters of spike rising phase are evaluated as amperometric signal rises from 35% to 90% of the peak amplitude. The time duration and slope of the linear fitting of this segment is defined as the spike rise time (t_{rise}) and the rise slope, respectively. The foot parameters were only calculated from the footed-amperometric signals which have an amplitude greater than 20 pA because foot can only be definitely resolved for events with relatively large amplitude (Wang et al., 2006). Foot probability is obtained by calculating the percentage of the events that have a preceding foot. I_{foot} , t_{foot} and Q_{foot} are calculated from the pre-spike foot segment, whose beginning is defined as the time point when the current signal exceeded the baseline noise while ending defined as extrapolation of linear fitting of the rising phase to the baseline. The time duration of the foot is quantified as the foot time (t_{foot}) and the area of this region as

foot charge (Q_{foot}). The foot amplitude (I_{foot}) is calculated as the plateau value of the exponential fitting (Fig. 1.6) (Mosharov & Sulzer, 2005).

Statistics of the spike parameters is represented by mean $\pm$ SEM (standard error of the mean). Statistical significance is evaluated with Student's t -test for normal distributed data. For the data with skewed distribution, Kolmogorov–Smirnov test is applied.

2.5 Total internal reflection fluorescent microscopy (TIRFM) imaging

2.4.1 Fluorescence labeling of large dense core secretory vesicles (LDCV) in PC12 cells

To be visualized under TIRFM, the vesicles require fluorescent labeling. Acridine orange that aggregates in acidic compartment (Oheim et al., 1998a) has been used to stain secretory vesicles. But it is not specific as acridine orange also stains lysosomes and other acidic organelles. Other dyes such as FM1-43 (Zenisek et al., 2002) have also been utilized to non-selectively label secretory vesicles. Most of these dyes can only provide weak fluorescent signals and are often sensitive to photo-bleaching. We labeled the LDCVs in PC12 cells by EGFP-tagged neuropeptide-Y (NPY-EGFP). NPY is a hormone molecule compartmentalized in LDCVs (Schneckenburger, 2005). NPY-EGFP plasmid (a kind gift from Dr. Wolf Almers, Vollum Institute, Oregon Health Sciences University) was transfected into PC12 cells 2-3 days prior to TIRFM experiments.

2.4.2 Experimental protocols

The TIRFM imaging was conducted using a microscope of Axiovert 200M series (Carl Zeiss Inc., Germany) equipped with a 100×1.45 NA TIRF objective lens. EGFP was excited by 488-nm laser, and the emission wavelength was collected at 520 nm. The angle of the laser beam is adjusted manually to optimize the thickness of the evanescent field until satisfied images of green fluorescence labeled vesicles can be obtained.

Fig. 2.3 TIRFM imaging of LDCVs in PC12 cells. The cell contour is outlined by dashed lines. The NPY-EGFP labeled vesicles are indicated by circles. The inset shows a typical trajectory of a single vesicle. The dashed and arrowed line indicates the LCD. The dashed rectangle defined by LCD and SCD just encases all the vesicle footprints.

Time-lapse digital images were acquired with exposure time of 20 ms and 2-Hz sampling frequency by a CCD camera controlled by MetaMorph 6.3 program (Molecular Devices, Downingtown, PA, USA). The imaging chamber was maintained at 25°C throughout the experiment. Cells, which appear unhealthy or are apparently differentiated or are clustered or have less than ten fluorescently labeled vesicles, are considered not typical and thus not chosen for experiments.

2.4.3 Analysis of vesicle motion

Vesicles were individually tracked using either MetaMorph or Image J (an open-source Java-based image processing program developed at National Institute of Health, <http://rsb.info.nih.gov/ij/>). The vesicle velocity, dwell time, and coverage diameters were obtained by a program based on Igor (WaveMetrics, Lake Oswego, OR, USA). We define the average velocity as the total distance of vesicle motion divided by vesicle dwell time. The longest distance between any two vesicle footprints is defined as the long coverage diameter (LCD), while the sum of the longest perpendicular distances to the LCD axis from two footprints at each side is defined as the short coverage diameter (SCD) (Fig. 2.3). Statistical analyses, namely, SEM and Student's *t*-test were performed to assess cell-to-cell variation.

2.5 Intracellular free calcium concentration ($[Ca^{2+}]_i$) imaging

2.5.1 Ca^{2+} indicator

The level of $[Ca^{2+}]_i$ was reported by a membrane-permeable and Ca^{2+} -sensitive dye, fura-2 acetoxymethyl ester (fura-2-AM, Molecular Probes, Eugene, OR, USA). Fura2-AM is a ratiometric dye that allows for Ca^{2+} calculation based on a ratio of measurements made with either two excitation or two emission wavelengths. Fura-2 undergoes a shift in

excitation spectrum upon Ca^{2+} binding (Fig. 2.4). The $[\text{Ca}^{2+}]_i$ concentration in fura-2 loaded cell can be estimated by measuring the ratio of emitted fluorescence at 510 nm in response to two excitation wavelengths (340 nm and 380 nm). This measurement is only dependent on Ca^{2+} concentration but not on the absolute amount of fluorescence and other background signals such as from cell autofluorescence. In contrast to non-ratiometric measurement which based on absolute emission intensity, this method is insensitive to dye loading, cell thickness, photo-bleaching, and dye leakage (Grynkiewicz et al., 1985).

Fig. 2.4 The excitation spectrum of fura-2-AM (Molecular Probes, Eugene, OR, USA).

Moreover, the indicator used in our study is acetoxymethyl (AM) ester of fura-2, which is membrane permeable. Cells can uptake AM esters by incubation with dilute aqueous solutions without any necessity to invasively disrupt the membrane. Nonspecific esterases present in cytosol then hydrolyze the AM esters to release the polyanionic fura-2 which will be trapped inside the cell. The final intracellular concentration of the

liberated Ca^{2+} -sensitive indicator can be much higher than the incubation concentration as the leakage rate is very low. This allows the real time recording of $[\text{Ca}^{2+}]_i$ in intact living cells (Tsien et al., 1984), whereas the impermeant form of fura-2 requires cell permeabilization or microinjection to take effect.

2.5.2 The photometry system

The fluorescence is excited with a Polychrome V monochromator (TILL Photonics, Munich, Germany). The monochromator can produce any wavelength between 320 nm and 600 nm, simply controlled by an input command voltage between -10 V and +10 V with a switching speed between wavelengths < 2 ms (Fig. 2.5B).

The emitted light from the fluorescent probe travels through the inside of the microscope to the dichroic mirror located below the objective lens (Fig. 2.5C). This mirror (Fig. 2.5D) reflects shorter wavelengths whereas longer wavelengths pass through. We used a dichroic mirror with a cutoff wavelength of 505 nm (Omega optical 505DCXRU). This is designed so that the excitation wavelengths (< 505 nm) will be reflected up through the objective to the sample. Fluorescent light emitted from the sample (> 505 nm) will pass back through the objective and pass straight through the dichroic mirror and make its way to the photodetector. The photodetector (Fig. 2.5E) picks up the emission light and converts it to a voltage signal which is sampled and filtered by the EPC-10 patch-clamp amplifier.

Fig. 2.5 The setup of the photometry system. **A.** An overview of photometry system for single cell study. **B.** Polychrome V monochromator (TILL Photonics, Munich, Germany). **C.** Light path through the Olympus 40× objective. **D.** Inverted picture of the light path at the dichroic mirror. **E.** The TILL photodetector.

2.5.3 Experiment protocol for Ca^{2+} imaging

PC12 cells were incubated with 5 μM fura-2-AM in culture medium for 45 min at the normal culture condition. The cells were then washed and allowed to recover for at least 30 min in the bath solution prior to $[\text{Ca}^{2+}]_i$ imaging. The Ca^{2+} signal was induced by local perfusion of high K^+ solution for 60 s to the target cell. Using the Polychrome V monochromator, fura-2-AM was excited at 340 nm and 380 nm. The emission fluorescence was collected at 510 nm at 1 s interval. The intracellular Ca^{2+} concentration is reported by the fluorescence intensity ratio resulting from the two excitation wavelength (F340/F380).

2.6 Confocal microscopy

The actin organization is observed by confocal microscopy which can provide high-resolution 3D images from a cell.

PC12 cells were first fixed in phosphate buffer solution (PBS) (Gibco) containing 3.7% formaldehyde (Sigma) for 10 minutes followed by permeabilization with 0.1% Triton X-100 (Sigma) in PBS for 10 minutes and subsequent blocking with 1.5% bovine serum albumin (BSA) (Sigma) in PBS for 1 hour at room temperature. After thorough washing by PBS, the cells were incubated in 1:1000 (v/v) of Alexa635 conjugated phalloidin (Molecular Probes) in PBS containing 1.5% BSA for 40 min in the dark at room temperature to immune-stain F-actin. The stained actin networks were imaged using a

Zeiss LSM 510 Meta confocal laser scanning microscope (Carl Zeiss) with a 63× oil-immersed objective. The percentage of cells showing discontinuous rings of cortical F-actin (as the evidence of disassembly of cortical actin networks) was calculated as mean $\pm$ SEM.

CHAPTER 3

SIMULATION AND ANALYSIS OF VESICLE LATERAL MOVEMENT

3.1 Introduction

Direct observation of vesicle dynamics became possible with the emergence of total internal reflection fluorescence microscopy (TIRFM), which enables real-time visualization and tracking of individual fluorescent-tagged secretory vesicles (Holz, 2006, Allersma et al., 2004, Steyer et al., 1997). Characterization of vesicle trafficking has recently attracted much attention because the dynamics of vesicle motion may reflect the molecular state and the fusion competence of the vesicle. Large dense core vesicles (LDCVs) in neuroendocrine cells and synaptic vesicles in neurons undertake constant lateral movement within the subplasmalemmal region and vertical transistion between the inner cytosol and the subplasmalemmal region. Three types of vesicle lateral movement have been identified: confined Brownian motion, free Brownian motion and directional motion (Oheim & Stuhmer, 2000, Konopka & Weisshaar, 2004).

Evolvment of mean square displacement (MSD) (defined in Equation 3.1) over time has been proposed to distinguish the three types of vesicle motion (Nofal et al., 2007, Huet et al., 2006, Li et al., 2004), specifically, positively curved MSD curve indicates directional

motion, linear MSD curve indicates free Brownian motion, and negatively curved MSD curve indicates confined Brownian motion.

$$MSD(n\Delta t) = \frac{1}{N-n} \sum_{i=1}^{N-n} \{[x(j\Delta t + n\Delta t) - x(j\Delta t)]^2 + [y(j\Delta t + n\Delta t) - y(j\Delta t)]^2\} \quad \text{Equation 3.1}$$

However, MSD does not straightforwardly reflect the characteristics and physical parameters of motion. And MSD analysis alone does not provide information regarding the velocity, area of motion, etc. We established a new framework to analyze vesicle lateral motion. Here, the method was demonstrated and compared to MSD analysis based on Monte Carlo simulation. In Chapter 4, it was used to analyze vesicle trafficking in PC12 cells, whereby distinct populations of subplasmalemmal vesicles were identified by their characteristic velocity, dwell time, and confinement.

3.2 Monte Carlo Simulation of vesicle motion

Monte Carlo simulations of three different types of vesicle motion (free Brownian, confined Brownian and directional movement) and analysis of the simulation results were performed using programs we developed in Igor Pro (WaveMetrics, Lake Oswego, OR).

3.2.1 Simulation of free Brownian motion

Trajectory of vesicle motion were simulated as particle random walk, assuming the particle travels the same distance (unit step length = δ) during each discrete and minute time interval (τ) but in a (random) direction that is independent from the direction in the previous time interval. It can be mathematically proved that such simulation mimics the true Brownian random walk and the diffusion coefficient $D = \delta^2/(2\tau)$ (Berg, 1983).

The initial vesicle position is set to be at the origin. The motion direction (represented by a unit vector $\hat{u}$) is randomly computer-generated at every time interval. Then the next vesicle position is calculated as show in Equation 3.2.

$$Position(x+1, y+1) = position(x, y) + \delta \times \hat{u} \quad \text{Equation 3.2}$$

$\hat{u}$: computer - generated unit vector

An example trajectory of free vesicle motion which comprises of 100 steps of random walk is depicted in Fig. 3.1A. We define the longest distance between any two vesicle footprints as the long coverage diameter (LCD) (Fig. 3.1A). The sum of the longest perpendicular distances to the LCD axis from two footprints at each side is defined as the short coverage diameter (SCD). All the vesicle footprints fall into the rectangular area specified by the long and short coverage diameters, which gives the first order estimation of the area coverage of vesicle motion.

Fig. 3.1 Simulated free Brownian motion (total time = 100 τ). **A.** A representative trajectory of simulated vesicle movement. The arrowed line indicates the LCD while the two dashed and arrowed lines indicate the SCD. The dashed rectangle defined by LCD and SCD is the first order estimation of the coverage of vesicle motion. **B. and D.** Evolvment of LCD (B) and MSD (D) over time. **C. and E.** The LCD (C) and MSD (E) curves averaged from 100 simulations. The average LCD curve (C, open circle) is fitted by the equation given in Equation 3.3 (C, line).

The evolvement of LCD over time of the example vesicle movement (Fig. 3.1A) is plotted in Fig. 3.1B. The curve clearly shows how the vesicle travels away from the initial position over time. According to Einstein's theory, LCD should diverge towards infinite as time increases, in a pattern governed by Equation 3.3. Indeed, the average LCD curve averaged from 100 simulations (Fig. 3.1C) can be fitted by equation 3.3. And the fitting gives a diffusion coefficient of $D = 0.45 \delta^2/\tau$, close to the true value of $0.5 \delta^2/\tau$.

$$LCD = \sqrt{4Dt} = \delta \sqrt{2t/\tau} = v \sqrt{2\pi} \quad \text{Equation 3.3}$$

MSD of the vesicle motion depicted in Fig. 3.1A increases linearly (Fig. 3.1D) confirming the free Brownian nature of the vesicle motion (Nofal et al., 2007). The MSD curve averaged from 100 simulations show the similar trend (Fig. 3.1E). Other than determining the type of vesicle motion, however, limited information can be extracted from such analysis.

3.2.2 Simulation of confined Brownian vesicle motion

The confined Brownian motion is similarly simulated as described above, except that a circular boundary (cage) is imposed to confine the range of vesicle motion. The boundary is reflective, meaning when the vesicle hits the boundary it bounds back with an angle (θ) symmetric to the incident angle with reference with the radial vector ($\hat{n}$) pointing to the origin. In this case, the next position is calculated by Equation 3.4 where x_1 is the current position, x_1' is the next position, x_0 the bouncing point.

Fig. 3.2 Simulated restricted Brownian motion (cage diameter = 4δ , total time = 100τ). **A.** A representative trajectory of a simulated vesicle is depicted. **B. and D.** The LCD (B) and MSD (D) of the trajectory shown in A is depicted versus time, respectively. **C. and E.** The LCD (C) and MSD (E) over time curves averaged from 100 simulations. The averaged LCD over time curve (C, open circle) is fitted by double-exponential (C, line), giving a plateau value = 3.85δ , $\tau_{\text{fast}} = 3.1\tau$ and $\tau_{\text{slow}} = 19.1\tau$.

An example of simulated trajectory of confined Brownian motion with a cage size of 4δ is shown in Fig. 3.2A. The LCD curve of this movement (Fig. 3.2B) approaches quickly to the plateau level indicating a boundary that the vesicle cannot cross. The average LCD curve (Fig. 3.2C, from 100 simulations) can be fitted by a double-exponential with a plateau level corresponding to the cage size (4δ). Such LCD analysis can be used to estimate the size of the physical barrier that confines vesicle motion. In addition, the time constants by which LCD approaches the cage size reflect, although indirectly, the velocity of the vesicle movement. As expected from confined Brownian motion, the MSD curve of the example trajectory (Fig. 3.2D) and the average MSD from 100 simulations (Fig. 3.2E) exhibit a negative or downward curvature.

3.2.3 Simulation of directional motion

Vesicles sometimes travel directionally in addition to random walk, likely driven by motor protein myosin along an actin track. In our simulations, the motion direction is determined by two components: a defined directional component (30%) and a random component (70%).

Fig. 3.3 Simulated directional motion (total time = 100 τ). **A.** A representative trajectory **B. and D.** The LCD (B) and MSD (D) curves of the trajectory shown in A. **C. and E.** The LCD (C) and MSD (E) curves averaged from 100 simulations. The average LCD curve (C, open circle) is fitted by a line (C, line).

The computational method for the unit vector to apply in Equation 3.2 is shown in Equation 3.5.

$$\text{unit vector for directional motion } \hat{u} = \frac{0.7 \times \hat{r} + 0.3 \times \hat{d}}{\sqrt{0.7^2 + 0.3^2}} \quad \text{Equation 3.5}$$

$\hat{r}$: randomly generated, $\hat{d}$: unit vector of $\frac{\pi}{4}$

Figure 3.3A shows a typical trajectory of a simulated vesicle motion with 100 walk-steps. And its LCD is nearly linear. LCD curve averaged from 100 simulations can be fitted by a line, the slop of which is the velocity along the directional movement.

3.3 Analysis of vesicle trafficking in PC12 cells

LDCVs in PC12 cells can be specifically labeled by overexpressing neuropeptide Y fused to enhanced green fluorescence protein (NPY-EGFP). We track individual vesicles under TIRFM and calculate the instantaneous velocity (the distance that the vesicle travels between two successive images divided by the time interval), the vesicle dwell time (from beginning of the imaging or its first appearance during the imaging to the end of the imaging or its disappearance during the imaging), values of LCD and SCD and area at every time point. Fig. 3.4 presents the motion trajectories of three subplasmalemmal vesicles. These vesicles have distinct motion characteristics which relates to their particular molecular state and fusion competence.

Fig. 3.4 Motion trajectories of three vesicles in a typical PC12 cell.

CHAPTER 4

EFFECTS OF PHORBOL ESTER ON VESICLE DYNAMICS AND EXOCYTOSIS

4.1 Introduction

As reviewed in Chapter 1, exocytosis is a complex process, including vesicle trafficking, docking, priming, and final fusion, highly regulated through the orchestrated actions of various proteins and lipids. Diacylglycerol (DAG), a secondary messenger lipid generated by phospholipase C (PLC) dependent cleavage of phosphatidylinositol biphosphate in response to a variety of biological signals, modulates multiple stages along exocytotic cascade through various mechanisms (Roldan & Harrison, 1992, Wenk & De Camilli, 2004, Davletov et al., 2007).

It has long been recognized that DAG potentiates the release of neurotransmitters at neuronal synapses and hormone release from endocrine cells through activation of protein kinase C (PKC) (Gillis et al., 1996, Yang et al., 2002, Stevens & Sullivan, 1998, Morgan et al., 2005). A number of proteins which involve in different aspects of exocytosis have been identified as PKC substrates (reviewed in Chapter 1). More recently, it has been revealed that, in parallel to its classic action on PKC, DAG also binds with Munc13 and consequently induces augmentation of exocytosis (Basu et al., 2007). Munc13 is believed to be an essential priming protein which renders vesicle fusion

competence by assisting in the assembly of the functional SNARE complex (Kwan & Gaisano, 2007, Rhee et al., 2002). PKC dependent and independent pathways converge on exocytosis machinery in a collaborative and mutually dependent manner (Wierda et al., 2007).

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

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

As described in the last chapter, LDCVs in PC12 cells were labeled by NPY-EGFP and observed under TIRFM. Time-elapsd images were taken over two minutes at 0.5 seconds time interval to reveal vesicle dynamics. Vesicles in the evanescent field did not dock on the plasma membrane stably as previously assumed as a prelude to fusion. Instead, they generally manifested constant, but apparently confined, lateral motion, as well as vertical trafficking (transition between the inner cytosol and subplasmalemmal

region). Vesicles in a cell were individually tracked and analyzed to obtain information about their trajectories, velocities, and dwell time.

Fig. 4.1
Subplasmalemmal vesicles can be classified into three populations: visiting vesicles (blue), mobile pre-docked vesicles (red) and sedentary pre-docked vesicles (green). And their motion kinetics was differentially modulated by PMA and BIS. **A.** The scatter plot of vesicle dwell time versus average velocity. Each dot represents one vesicle. There are total of 495 vesicles from 10

cells. **B.** An example trajectory of a mobile pre-docked vesicle is depicted. Color scales with time in a duration of 46.5 seconds. The motion coverage can be described by LCD (red arrowed-and-dashed line) and SCD (the total length of the two black arrowed-and-dashed lines). **C.** Each trace represents how a vesicle LCD evolves with time. All the 40 vesicles are from the same cell. **D.** LCD curves are averaged from: 50 visiting vesicles in 10 control cells (blue circles); 104 visiting vesicles in 9 PMA treated cells (blue squares); 25 visiting vesicles in 7 PMA and BIS treated cells (blue triangles); 74 mobile pre-docked vesicles in 10 control cells (red circles); 70 mobile pre-docked vesicles in 9 PMA treated cells (red squares); 43 mobile pre-docked vesicles in 7 PMA and BIS treated cells (red triangles); 77 sedentary pre-docked vesicles in 10 control cells (green circles); 48 sedentary pre-docked vesicles in 9 PMA treated cells (green squares); 84 sedentary pre-docked vesicles in 7 PMA and BIS treated cells (green triangles). Each curve is fitted by a double-exponential whose approaching limit predicts the cage size of the vesicle motion.

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

Figure 4.1A illustrates the scatter plot of dwell time versus average velocity, in which each dot represents one vesicle (the total of 495 vesicles from 10 cells). Average velocity is defined as the total distance of vesicle motion divided by vesicle dwell time. It is evident that vesicles were clustered into three groups. The visiting vesicles (Fig. 4.1A, blue) were short lived with an average dwell time of 7.9 ± 0.77 seconds and moved rapidly with an average velocity of 0.74 ± 0.024 $\mu\text{m/s}$. The mobile pre-docked vesicles (Fig. 4.1A, red) lingered in the near plasma region 5 times longer than the visitors (43.3 ± 3.09 seconds) and with a much slower velocity (0.39 ± 0.021 $\mu\text{m/s}$). The sedentary pre-

docked vesicles (Fig. 4.1A, green) were most stably leashed onto the subplasmalemmal membrane region with the slowest mobility ($0.16 \pm 0.014 \mu\text{m/s}$).

In agreement with the previous studies (Johns et al., 2001, Oheim & Stuhmer, 2000, Nofal et al., 2007), vesicle motion was found to be highly restricted as if vesicles were confined in cages. An example trajectory of a mobile pre-docked vesicle is depicted in Fig. 4.1B. As described in Chapter 3, the longest distance between any two vesicle footprints is defined as the long coverage diameter (LCD) (Fig. 4.1B, arrowed-and-dashed red line) while the short coverage diameter (SCD) stands for the sum of the longest perpendicular distances to the LCD axis from two footprints at each side (Fig. 4.1B, two arrowed-and-dashed black lines). The rectangular area specified by the LCD and SCD contains all the vesicle footprints.

In Fig. 4.1C, each trace demonstrates how LCD of a vesicle evolves with time (from its appearance to disappearance) in a typical cell. Visiting vesicles, mobile pre-docked vesicles and sedentary pre-docked vesicles, which are represented by different colors (blue, red and green, respectively), showed distinct kinetics. As described in last chapter, if a vesicle undergoes unbounded Brownian random walk, its motion coverage would diverge over time, i.e., $LCD \propto \sqrt{t}$. On the contrary, vesicle LCD approaches a limit in a time scale of ten seconds, suggesting that vesicle motion is confined within small cages delimited by certain physical barriers or due to some tethering forces. The traces from different types of vesicles are well separated, indicating characteristic differences in their motion.

The average behaviors of the three vesicle pools are obtained from 10 cells (Fig. 4.1D). Due to varying dwell time, we average the LCD curves of the visiting vesicles over the initial 10 seconds, only for those which survived up to 10 seconds or more (blue circles; the average from 50 vesicles). Mobile pre-docked vesicles are averaged over 20 second time frame, while those whose dwell time were less than 20 seconds are not used (red circles; 74 vesicles). Sedentary pre-docked vesicles are also averaged over 20 seconds (green circles; 77 vesicles). The 10 second and 20 second time frames are selected for averaging to balance two considerations: first, to assure that not too many vesicles are left out for analysis; on the other hand, only vesicles staying long enough to reflect the motion trend should be chosen for analysis. Clearly from their averaged LCD curves, the three types of vesicles differ in their motion kinetics.

In contrast to the case of unlimited Brownian random walk in which LCD curve would diverge with $\sqrt{t}$, the averaged vesicle LCD curves can be well fitted by a double-exponential. And they converge to a limit rapidly with a fast time constant of ~ 0.5 s and a slower time constant of ~ 10 s (Fig. 4.1D). The approaching limit predicts the ultimate boundary (cage size) of vesicle motion regardless of how long the vesicle remains in the subplasmalemmal membrane region before losing into the cytosol and how fast the vesicle moves. The time constants, on the other hand, reflect the mobility of the vesicle. Specifically, the averaged LCD curve of the visiting vesicles approaches (as $t \rightarrow \infty$) the cage size of $1.60 \mu\text{m}$ with an initial fast kinetics (time constant $\tau_1 = 0.45$ s) followed by a slower kinetic component (time constant $\tau_2 = 7.74$ s). Comparing to the visiting vesicles,

the sedentary pre-docked vesicles showed a much smaller cage size ($0.69\ \mu\text{m}$) and slower kinetics ($\tau_1 = 0.77\ \text{s}; \tau_2 = 15.6\ \text{s}$), whereas the mobile pre-docked vesicles showed a moderate cage size ($0.98\ \mu\text{m}$) and moderate kinetics ($\tau_1 = 0.38\ \text{s}; \tau_2 = 13.76\ \text{s}$).

4.3 Vesicle lateral motion is modulated by PMA

When the cells ($n = 9$) were treated with PMA, interestingly, the motion of the visiting vesicles became confined to a much greater extent with a reduced cage size of $1.0\ \mu\text{m}$ (blue squares in Fig. 4.1D; averaged from 104 vesicles). To test the roles of PKC isoforms in this effect, bisindolylmaleimide (BIS) which is a specific PKC inhibitor through competitive inhibition of ATP binding within the PKC catalytic domain was applied in addition to PMA treatment (7 cells). BIS should not affect non-kinase phorbol ester / DAG-binding proteins such as Munc13. As shown in Fig. 4.1B, BIS (7 cells) was not able to relief the PMA induced further motion restriction (blue triangles; 35 vesicles). Hence it is likely that PMA exaggerates the confinement of the visiting vesicle's lateral motion through PKC independent mechanisms. By contrast, PMA treatment only slightly reduced the cage size of the sedentary pre-docked vesicles to $0.55\ \mu\text{m}$ (green squares in Fig. 4.1D; 70 vesicles); and did not affect the motion of the mobile pre-docked vesicles appreciably (red squares in Fig. 4.1D; 48 vesicles). Whereas, additional application of BIS significantly reduced the cage sizes of both the sedentary and mobile pre-docked vesicles to $0.45\ \mu\text{m}$ (green triangles in Fig. 4.1D; 84 vesicles) and $0.53\ \mu\text{m}$ (red triangles in Fig. 4.1D; 43 vesicles), respectively. Intricately, these results imply that although

boosting PKC activation by PMA does not impact the movement of pre-docked vesicles significantly, inhibition of PKCs leads to severe motion confinement.

Fig. 4.2 Vesicle velocity was modulated by PMA and BIS. 10 control cells, 9 PMA treated cells, 7 PMA and BIS treated cells are used for the statistics. Data are shown as mean $\pm$ SEM. Student's *t*-test: ** $p < 0.01$ vs. control.

Comparing the velocities and the cage sizes of the three types of vesicles, it is noted that less mobile vesicles tend to be imprisoned within a smaller area. In addition, vesicle velocities of the three types of vesicles responded to the application of PMA and BIS similarly as the induced changes in their cage sizes, as demonstrated in Fig. 4.2. For example, PMA reduces both the cage size and the velocity of the visiting vesicles. It is conceivable that vesicle mobility and how far the vesicle can travel may be determined by the same tethering or confining mechanisms.

4.4 PMA reduces the number of unreleasable sedentary pre-docked vesicles

Sedentary vesicles made up ~35% of all pre-docked vesicles (Fig. 4.3A, 10 cells). PMA treatment (9 cells) significantly lowered this percentage to ~14%. This reduction was

reversed by BIS (7 cells), giving rise to ~51% and hence implying the involvement of PKCs. The sedentary pre-docked vesicles were generally brighter than other vesicles. Under total internal reflection illumination, the fluorescence brightness is exponentially inverse-proportional to the distance between the fluorescent vesicle and the membrane-coverslip interface. Therefore, sedentary vesicles are more closely attached onto the plasma membrane. But somewhat surprisingly, these vesicles are often reluctant to be released upon triggering. The left panel in Fig. 4.3B is the last TIRFM image of a two-minute recording on a control cell. The sedentary pre-docked vesicles which stayed on throughout the two-minute recording are identified and indicated by arrows. The right panel in Fig. 4.3B shows the TIRFM image of the same cell after the introduction of high K^+ solution for 48 seconds. High K^+ solution is commonly used to depolarize the cell membrane and subsequently induce exocytosis. All the sedentary pre-docked vesicles remained after high K^+ stimulation, whereas disappearance of the other vesicles was observed.

Subject to high K^+ stimulation, the averaged release time courses of the pre-docked vesicles in three conditions (14 control cells, 13 cells with PMA treatment, and 7 cells treated with PMA plus BIS) are shown in Fig. 4.3C. The percentages of the remaining (unreleased) vesicles are consistent with the percentages of the sedentary vesicles shown in Fig. 4.3A. It can be speculated that the sedentary vesicles are in a docked but unprimed state. And PKC activation by PMA primes these vesicles to the readily releasable state, *via* phosphorylation of secretory proteins such as Munc18 (Nili et al., 2006) and SNAP-

25 (Yang et al., 2007). In comparison to sedentary vesicles, mobile pre-docked vesicles are essentially readily releasable upon stimulation.

Fig. 4.3 PMA reduced the number of unreleasable sedentary pre-docked vesicles. **A.** The percentage of sedentary pre-docked vesicles in all pre-docked vesicles was significantly reduced by PMA (13 cells) comparing to the control (14 cells). And BIS (7 cells) reversed the PMA effect. Data are shown as mean $\pm$ SEM. **B.** Sedentary vesicles were reluctant to be released. The left panel shows the last TIRFM image after a 2-minute recording. Sedentary pre-docked vesicles are identified by arrows. The right panel shows the last TIRFM image after 48 seconds in presence of high K^+ stimulation. All the sedentary pre-docked vesicles (indicated by arrows) remained while disappearing of other vesicles was evident (highlighted by circles) showing in the left panel were released. The squares indicate two recently arrived vesicles. The scale bar corresponds to 5 μm . **C.** The percentage of the remaining vesicles in presence of high K^+ stimulation was largely decreased by PMA treatment (squares, 13 cells) comparing to the control (circles, 14 cells). On the other hand, BIS reversed such PMA effect (triangles, 7 cells). Student's t -test: ** $p < 0.01$ vs. control.

4.5 PMA enhances exocytosis mainly by increasing the delivery of visiting vesicles

Phorbol ester is known to enhance exocytosis in various cell types. The mechanisms underlying, however, are not fully understood yet. In some studies, it has been attributed to the enhancement of vesicles number close to the plasma membrane as a result of PKC activation based on electron microscopy observations (Gil et al., 2000, Danks et al., 1999). From TIRFM imaging on live PC12 cells, we however found that, PMA did not increase the total number of the visible vesicles in subplasmalemmal membrane region at any given snapshot in time (average from 6 cells, open squares in Fig. 4.4A). On the other hand, the arrival rate of visiting vesicles was dramatically increased by PMA (6 cells, filled squares in Fig. 4.4A). PKC activation is likely responsible for this increased vesicle transport from the cytosol, because it can be completely blocked by the application of BIS (7 cells, filled triangles in Fig. 4.4A).

PMA enhancement in exocytosis was confirmed by carbon fiber amperometry (Fig. 4.4B). Carbon fiber microelectrode, with a diameter of 5 μm , can only detect vesicle release from the membrane area just underneath, which only accounts for a small fraction (~5-10%) of the total cell membrane area. Considering that the total number of pre-docked vesicles is typically less than 40, most of the exocytotic events recorded by carbon fiber must be produced by newly arrived visiting vesicles. Therefore, it can be concluded that the PMA enhancement reported by amperometry recording is achieved by increasing delivery of releasable visiting vesicles to the plasma membrane. Large portion of the PMA recruited visiting vesicles, however, are not fusion competent, since the PMA

induced increase (33%) in the amperometric signal is much less than the increase (230%) in the arrival rate of visiting vesicles as observed under TIRFM.

Fig. 4.4 PMA enhanced exocytosis by increasing the delivery of visiting vesicles in PKC dependent pathways. **A.** PMA application dramatically increased the delivery of visiting vesicles (filled squares averaged from 6 cells versus filled circles for 6 control cells) without appreciably affect the total number of the subplasmalemmal vesicles (open squares versus open circle for the control cells). BIS treatment (filled triangles, 7 cells) completely abolished the PMA enhancement in visiting vesicle delivery. **B.** Cumulative fusion events detected by carbon fiber amperometry demonstrates that PMA caused more exocytosis (line, 11 cells) than the control cells (dotted line, 22 cells) in response to high K^+ stimulation, whereas BIS (dashed line, 11 cells) completely eliminated the PMA enhancement. The right panel illustrates example amperometry traces from 3 representative cells. The black bars underneath each trace indicate 60-second stimulation of high K^+ solution.

4.6 Vesicle dwell time is reduced by PMA

Increased delivery of visiting vesicles does not cause accumulation of vesicles in the subplasmalemmal membrane region (Fig. 4.4A) because PMA also reduces the dwell time of both pre-docked vesicles and visiting vesicles, as if the interactions to keep the vesicles attaching to the membrane are weakened (Fig. 4.5). In other words, PMA does not alter the equilibrium between the vesicle recruitment and vesicle retrieval. PMA shortens vesicle dwell time probably through PKC dependent pathways as BIS treatment fully abolishes this effect.

Fig. 4.5 PMA decreased the dwell time of the visiting and pre-docked vesicles, and BIS effectively antagonized the PMA effect. The statistics are obtained from 10 control cells, 9 PMA treated cells, 7 PMA and BIS treated cells. Data are shown as mean $\pm$ SEM. Student's *t*-test: * $p < 0.01$ vs. control.

4.7 Vesicle motion coverage is regulated by PMA

As discussed above, PMA modulates boundary, velocity, and dwell time of vesicle motion. Consequently, the total coverage area of vesicle lateral motion, which can be

estimated by LCD multiplies SCD, are subject to PMA regulation. As seen in Fig. 4.6A, PMA largely shrunk the motion coverage of visiting vesicles and mobile pre-docked vesicles, but not that of sedentary pre-docked vesicles. BIS failed to antagonize PMA effect. Instead, it further reduced the motion coverage of mobile pre-docked vesicles and also caused decrease of the coverage of sedentary pre-docked vesicles. Demonstrated in Fig. 4.6B, both PMA and BIS, did not cause appreciable changes in the aspect ratios of motion coverage which is defined as SCD divided by LCD.

Fig. 4.6 PMA and BIS changed the coverage area of vesicle motion but not aspect ratio. The statistics are obtained from 10 control cells, 9 PMA treated cells, 7 PMA and BIS treated cells. **A.** PMA largely reduced the motion area of the visiting and mobile pre-docked vesicles, whereas BIS reduced the motion area of the mobile and sedentary pre-docked vesicles. **B.** Neither PMA nor BIS treatments altered the aspect ratio of the motion coverage (SCD / LCD). Data are shown as mean $\pm$ SEM. Student's *t*-test: * $p < 0.01$ vs. control.

4.8 Conclusion and discussion

Using TIRFM, we visualized individual secretory vesicles that are immediately adjacent to the plasma membrane in PC12 cells and analyzed their movement. We classify these

vesicles into three groups: visiting vesicles, mobile pre-docked vesicles, and sedentary pre-docked vesicles. These populations of vesicles differ in their motion characteristics, fusion competence, and dependence to PMA modulation, suggesting that they constitute molecularly heterogeneous vesicles.

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

Interestingly and perhaps counter-intuitively, sedentary pre-docked vesicles, which are most strongly attached onto the subplasmalemmal membrane region indicated by their lowest mobility, longest dwell time and high fluorescence brightness, are most fusion incompetent. It is plausible that tethering and docking interactions between vesicles and plasma membrane are actually fusion-inhibitory to prevent them from being constitutively released. In comparison, the mobile pre-docked vesicles are more loosely associated with plasma membrane. But they are fusion competent and may be responsible for the initial phase of release in response to intracellular Ca^{2+} elevation because of their proximity to the exocytotic site. Visiting vesicles arrived from the inner cytosol can only be transiently captured in the subplasmalemmal membrane region. Despite the high

lateral mobility, they are releasable, and in fact, account for the majority of the fusion events recorded by carbon fiber microelectrode. It has been demonstrated that these recruited vesicles can be immediately released once they reach the plasma membrane and they are responsible for the late and sustained phase of exocytosis (Mica et al., 2004).

In Chapter 3, we describe a new method to characterize vesicle motion which allows us to distinguish the motion characteristics of different vesicle populations and monitor kinetics of vesicle movement over time. Two parameters, termed as LCD and SCD, are introduced to simply quantify the coverage of vesicle lateral movement. The evolvement of LCD over time reflects the nature of vesicle motion. If vesicles undergo unbounded Brownian random walk, LCD would increase in proportional to square root of time. Specifically, $LCD = \sqrt{4Dt}$, where D is the diffusion coefficient of the vesicle (Berg, 1983). To illustrate this, we performed Monte Carlo simulations. In simulations, the time interval (τ) between random walk steps is 0.5 seconds, the same as the TIRFM imaging interval we used. The step size of random walk (δ) is a constant, which is determined based on an assumed vesicle velocity ($v = \delta/\tau$). The step size relates to the diffusion coefficient by equation: $D = \delta^2 / (2\tau)$ (Berg, 1983). An example of stimulated trajectory in 120 seconds duration is depicted in Fig. 4.7A, assuming the mean velocity of the mobile pre-docked vesicles ($v = 0.39 \mu\text{m/s}$). Fig. 4.7B exhibits the averaged LCD curves, each obtained from 100 simulations, by assuming the vesicle velocity of $0.16 \mu\text{m/s}$ (triangles), $0.39 \mu\text{m/s}$ (squares), and $0.74 \mu\text{m/s}$ (circles), respectively corresponding to the mean velocities of the sedentary pre-docked, the mobile pre-docked and the visiting vesicles. These curves agree well with theoretical predictions, i.e.,

$LCD = \sqrt{4Dt} = \delta \sqrt{2t/\tau} = v \sqrt{2\pi}$ (fitted solid curves). They diverge towards infinite as time increases. The actual vesicle motions are apparently discrepant from such unbounded Brownian random walk.

Fig. 4.7 Monte Carlo simulations of vesicle lateral motion at 0.5 seconds time interval. **A.** A stimulation of unbounded Brownian random walk in a duration of 120 seconds. The size of each random walk step is 0.195 μm corresponding to an instantaneous velocity of 0.39 μm/s. Scale bars indicate 1 μm in length. **B.** Averaged LCD curves. Each is obtained from 100 simulations of 20-second unbounded random walk by assuming a constant instantaneous velocity of 0.16 (triangles) or 0.39 (squares) or 0.74 μm/s (circles). Each LCD curve can be well fitted (solid curves) by the theoretical calculation (solid curves, see explanations in text). **C.** An stimulated caged Brownian random walk with the same duration and step size as in **a**. But a confining round boundary with a diameter of 0.98 μm is assumed. The scale bars correspond to 0.3 μm. **D.** Averaged LCD curves were obtained similarly as in **B**, except that a confine cage with a diameter of 0.69 (triangles) or 0.98 (squares) or 1.60 μm (circles) is assumed. The averaged vesicle LCDs in 10 control cells from Fig. 4.1D are re-plotted (dark curves) for comparison

In Fig. 4.7C, a random walk trajectory is obtained by repeating simulation procedure as in Fig. 4.7A while imposing a confining round boundary (a hypothetical cage). In this example, we assumed a diameter of $0.98\ \mu\text{m}$ for the confining cage, identical to the cage size of the mobile pre-docked vesicles. In Fig. 4.7D, three averaged LCD curves were obtained, each from 100 simulations, by assuming the mean velocities and the cage sizes of the three types of vesicles, respectively. These curves can be well fitted by a double-exponential (solid curves). And they agree very well with the actual experimental data shown in Fig. 4.1D (re-plotted in Fig. 4.7D as thick solid curves). These simulation results unambiguously demonstrate that the motion of subplasmalemmal vesicle is caged Brownian random walk. Nevertheless, some vesicles exhibited directed motion (travel directionally for a relatively long distance), deviating from their typical behavior. These events, however, are rare, particularly within the short time frames (10s or 20s) that we used for analysis.

The cortical actin meshwork probably is the dominant factor that hinders vesicle motion (Gil et al., 2000). This view was supported by the experiments using latrunculin A which disrupts actin cortex by preventing actin polymerization. Vesicle motion in latrunculin A treated cells is analogous to unbounded Brownian random walk. Interestingly, a recent study reported the existence of cytoskeletal polygonal cages in bovine chromaffin cells that physically confine the movement of the vesicle inside (Giner et al., 2007). The estimated sizes of the cortical cages and the interior cages are about $0.48\ \mu\text{m}$ and $0.64\ \mu\text{m}$ in diameter, comparable to the predicted cage sizes of the pre-docked vesicles in our study. It appears that the cage size is inversely proportional to the vesicle velocity. It is

possible that the same tethering or confining interaction may slow down the vesicle velocity and set the limits for vesicle motion as well.

Further supporting the notion that the three types of vesicles are molecularly heterogeneous, we show that PMA modulates these morphologically defined vesicle pools differentially. PMA has long been recognized as a potent exocytosis promoter. Phorbol ester enhancement in exocytosis has been attributed to the induced PKC activation and the resulting increase in the size of the readily releasable vesicle pool (RRP) (Gillis et al., 1996, Stevens & Sullivan, 1998). Mainly derived from the membrane capacitance measurement of exocytosis, the release of RRP is defined as the initial rapid phase in the exocytosis kinetics responding to a step-like increase in intracellular calcium concentration by calcium uncaging. Vesicles in RRP are considered to be appropriately docked and fully primed and thus ready to be released immediately upon triggering (Sorensen, 2004). As a prerequisite to be readily releasable, these vesicles are presumably in close proximity with the plasma membrane (< 25 nm away from the membrane) to assure the critical SNARE protein pairing, i.e., complexing between vesicle SNARE (VAMP) and target SNAREs (syntaxin and SNAP-25) (Jahn & Scheller, 2006). Therefore, vesicles in RRP must be closely attached to the plasma membrane and visible in evanescent field. In this study on PC12 cells, we, however, found that phorbol ester does not increase the number of vesicles within subplasmalemmal membrane region.

Although fail to increase the number of docked vesicles, PMA facilitates priming and subsequent release of sedentary pre-docked vesicles likely through PKC phosphorylation

directly on fusion proteins. In PC12 cells, because the number of docked vesicles is small (typically, 20-40 vesicles), such facilitation in exocytosis is not significant. But for chromaffin and neuron cells which has hundreds of morphologically docked vesicles, such PMA facilitation is prominent and may underlie the observed increase in RRP. As demonstrated by our experiments, in PC12 cells, PMA enhances exocytosis mainly by increasing the rate of vesicle delivery to the plasma membrane, also in a PKC dependent manner.

Neuroendocrine cells possess a dense actin meshwork in the subplasmalemmal region, which serves as a physical barrier separating the reserved vesicles in the cytosol and the membrane-docked vesicles. On the other hand, F-actin can act as an active regulator through restricting the subplasmalemmal vesicles in position and driving the final membrane fusion (Eitzen, 2003). It has been documented that PKC activation (e.g., by PMA) causes disassembly and re-organization of cortical F-actin, clearing the way for vesicle transportation from the reserve pool in the cytosol to the plasma membrane along the remaining fine actin filaments (Trifaro et al., 2002). PKC activation disrupts and re-organizes actin networks probably through multiple pathways, including recruitment of actin severing protein scinderin (Eitzen, 2003) and phosphorylation of MARCKS (Park et al., 2006, Trifaro et al., 2002) or phosphorylation of other actin filament associated proteins (Larsson, 2006). It explains our observation that PMA largely increased the traffic between the reserved vesicle pool and docked vesicle pool. Specifically, both the delivery rate of visiting vesicles and the retrieval of cortical vesicles back into the cytosol were increased after PMA application. PKC specific inhibitor, BIS, completely abolished

this increased vesicle traffic. Nonetheless, such an actin centric view is perhaps too simple to picture the likely complicated mechanisms underlying. For example, increased trafficking may be resulted from the combined actions of multiple PKC effector proteins that participate in vesicle tethering and docking, for instances, Munc18 and Rab3 (Becherer & Rettig, 2006, Izumi et al., 2007, Barclay et al., 2003).

Seemingly contradictory to its disrupting action on actin meshwork, PMA causes restriction on vesicle motion, particularly on visiting vesicles. Unlike other actin disassembly drugs such as latrunculin A, PMA only reduces the density of actin meshwork instead of dissolving all the actin filaments. Therefore, PMA induced re-organization of actin cortex does not necessarily decrease the sizes of actin cages that confine vesicle motion. PMA restrains vesicle motion by decreasing its caging boundary and vesicle velocity, possibly through phorbol ester / DAG receptor proteins, other than PKCs such as Munc13, that involve in vesicle tethering, docking and priming. The overall consequence of reduced vesicle motion and dwell time by PMA is the significant reduction in the coverage area of vesicle motion. This is consistent with the finding from a recent paper (Nofal et al., 2007) which showed that both PMA treatment and overexpression of Munc13 largely eliminated long-range vesicle motion. But the authors in that study did not report PMA induced decreases in vesicle velocity and dwell time as we did, maybe because they used a different cell model – bovine chromaffin cells.

In summary, this study presents an approach to analyze the heterogeneous dynamics of secretory vesicles using total internal reflection fluorescence microscopy, and provides

further evidence that the PMA regulation in exocytosis is multifaceted and complex. This study demonstrates an analytical framework which, together with genetic manipulations, may allow quantitative assessment of the roles of specific secretory proteins in regulating various aspects of vesicle dynamics.

CHAPTER 5

MOLECLAR CLONING OF RECOMBINANT PLASMIDS FOR OVEREXPRESSION OF PKC ISOFORMS

5.1 Introduction

Pharmacological tools, such as PMA that acts as a protein kinase C (PKC) activator, and BIS as a PKC inhibitor, are usually used to study the functions of PKC. However, the exact mechanisms underlying the actions of PMA are often unclear due to the diversity of PKC isoforms and the caveats of pharmacological assessment. As mentioned in Chapter 1, PKC family consists of conventional PKCs (α , β , and γ), novel PKCs (δ , η , ϵ and θ), and atypical PKCs (ζ , τ , and λ). Seven different isoforms of PKC (conventional and novel) can be activated by PMA and thus isoform-specificity cannot be resolved simply based on PMA or BIS experiments. More intriguingly, PMA is also able to activate secretory proteins other than PKC such as Munc13.

Overexpression of wild-type (wt-) or dominant negative (dn-) forms of individual PKC isoforms by transfecting cells with plasmid coding for the protein of interest is instrumental to address isoform specificity. Overexpression of a particular PKC isoform amplifies its effects while overexpression of its inactive form suppresses its effects via competitive inhibition. A key issue of such strategy, however, is to assess the efficiency of plasmid transfection and subsequent protein expression. The FLAG-tag has been

widely used to label overexpressed proteins in plasmid transfection experiments. This is achieved via tagging the FLAG sequence onto either the 3' or 5' end of the gene of interest. But, visualization of FLAG-tag requires immunoblotting (Cenni et al., 2002) after cell fixing or lysis. Apparently, it is not suitable for studies on single living cells.

In our experiments, enhanced green fluorescent protein (EGFP) or *Discosoma* sp. Red (DsRed) was co-expressed with the PKC isoform from the same plasmid as a reporter. Under a fluorescent microscope, only cells lightened by such reporter as an indication of effective overexpression of the target protein were recorded. EGFP is the enhanced version of green fluorescent protein (GFP) which consists of 238 amino acids and was originally isolated from jellyfish *Aequorea victoria*. It gives green fluorescence when exposed to blue light, with an emission maximum at 507 nm and an excitation maximum at 488 nm. EGFP has brighter fluorescence, minor photo-toxicity and higher expression rate in eukaryotic cells comparing to conventional GFP (Tsien, 1998, Chalfie et al., 1994, Zhang et al., 1996). DsRed is a recently cloned fluorescent protein extracted from the coral *Discosoma* genus. As its maximal excitation and emission wavelengths (557 nm and 579 nm, respectively) are distant from that of EGFP, it works well as a co-reporter together with EGFP (Baird et al., 2000). In our TIRFM imaging experiments, DsRed was utilized because vesicles were labeled with NPY-EGFP.

In our scheme, the reporter fluorescence protein and PKC are expressed separately, because PKC function may be impaired if the reporter is directly conjugated onto it. To individually and concurrently express both the PKC isoform and the fluorescent protein,

we designed an internal ribosome entry site (IRES) between two open reading frames. The IRES is a nucleotide sequence that can initiate translation in the middle of a messenger RNA (mRNA), allowing translation of its downstream protein coding region through promoting the assembly of the ribosomal complex at the internal start codon of the mRNA independent of the 5'-cap (Jackson et al., 1990). Thus, the two individual proteins upstream and downstream of IRES can be translated concurrently from the same mRNA. In this study, eight recombinant plasmids ((wt/dn)-(PKC α /PKC ϵ)-IRES-(EGFP/DsRed)) were constructed to co-express the target protein (PKC α or PKC ϵ) and the reporter (EGFP or DsRed) using molecular cloning technique.

5.2 Plasmids construction

5.2.1 *The original plasmids and the strategy of subcloning*

The cDNA plasmids coding for wt-PKC α , dn-PKC α (K368N mutated), wt-PKC ϵ and dn-PKC ϵ (K437W mutated) were obtained from Dr. Alex Toker (Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA) through the Addgene plasmid collection (Cambridge, MA, USA). The dominant negative mutants of PKCs are permanently inactive in the ATP-binding site of C3 domain. The pIRES2-EGFP and pIRES2-DsRed-Express vector plasmids were purchased from Clontech (Mountain View, CA, USA).

Fig. 5.1 Original plasmids of pIRES2-EGFP and pIRES2-DsRed-Express. (Clontech co. ltd.)

The (wt/dn)-(PKC α /PKC ϵ)-IRES-(EGFP/DsRed) plasmids were constructed by subcloning the corresponding PKC cDNAs into pIRES2-EGFP or pIRES2-DsRed-Express vector at the sites of Nhe I and Sma I. Figure 5.2 summarizes the subcloning steps. Firstly, the original vector plasmid was digested at double restrict sites (Nhe I and Sma I, corresponding to A and B in Fig. 5.2) to obtain a cleaved-open vector (Fig. 5.2 white). Meanwhile, the original plasmid containing PKC gene was cleaved by the same double endonucleases to release the insert DNA fragment (Fig. 5.2 red). This fragment consisted of the entire open reading frame of PKC. Secondly, the insert DNA fragment and the cleaved-open vector DNA were purified using gel electrophoresis. Thirdly, the insert fragment was connected to the cleaved-open vector by the ligation of two cohesive ends (Nhe I and Sma I, corresponding to A and B in Fig. 5.2). Finally, the ligation products were amplified by transformation into bacteria (DH5 α line of *Escherichia coli*) and screened by antibiotics. The validity of the final DNA products was confirmed by gel electrophoresis and DNA sequencing.

Fig. 5.2 The steps of DNA subcloning. The original vector plasmid (top left) and the original plasmid containing insert DNA (top right) are digested at double restriction sites (A and B) to release a cleaved-open vector (middle left, white) and the insert fragment (middle right, red), both with two cohesive ends (A and B). Then the cleaved-open vector and insert fragment are connected by ligation of cohesive ends (A to A, B to B) to create a recombinant plasmid (bottom).

5.2.2 Digestion of the original plasmids by double restrict endonucleases

Double enzyme digestion of the original plasmids was performed using restrict endonuclease Nhe I and Sma I (New England Biolabs, Ipswich, MA, USA). The DNA molecules (0.3 ug/ μ l), endonuclease (0.2 a.u./ μ l) and bovine serum albumin (BSA) (0.1 μ g/ μ l) were mixed in NEB-buffer-4 in a total volume of 40 μ l and incubated at 37°C for 2 hours. Both of these two restrict endonucleases perform 100% activity in the NEB-buffer-4. BSA stabilizes enzymes during the digestion process and prevents adhesion of

the enzymes to the reaction tubes, without affecting the biochemical reaction. The double enzyme digestion can release linear DNA fragments with two cohesive ends (Nhe I and Sma I) at each terminal of the double strand.

5.2.3 Purification of the DNA fragments by gel electrophoresis

To separate the DNA fragments of interest from the products of enzyme digestion, gel electrophoresis was employed using a MGU-102T Horizontal Mini-Gel Kit equipped with an EPS-300 IIV Power Supply (C.B.S. Scientific, Del Mar, CA, USA). In brief, the digestion products were loaded to 0.8% agarose gel immersed in tris-acetate-EDTA (TAE) buffer (1st Base, Singapore, consists of 40 mM tris-base, 20 mM glacial acetate acid and 1 mM EDTA, titrated to pH 8.0). A potential of 80 V was applied to the DNA-loaded gel for 60 min. Driven by the electrical field, DNA molecules migrate in the gel, and smaller (or shorter) DNA molecules travel faster (Sambrook & Russell, 2001). GeneRuler 1kb DNA ladder (Fermentas, Ontario, Canada) was used to benchmark the size of the DNA molecule at each resulting band in the agarose gel. After electrophoresis, the gel was then stained for 10 min in room temperature in ethidium bromide and pictured under the BioDoc-it imaging system (UVP, Upland, CA, USA). The insert fragments of molecular size about 2~2.5 kb and open vectors of size about 5.2 or 5.3 kb can be identified. The corresponding DNA bands were then cut off from the gel. Subsequently the DNA fragments (both the insert section encoding for PKCs and cleaved-open vectors) were extracted from these bands and purified using the QIAquick Gel Extraction Kit

(Qiagen, Valencia, CA, USA). The purified linear DNA molecules were collected in de-ionized water for ligation.

5.2.4 Ligation of DNA fragments

Ligation of the insert fragment and the open vector extracted from the agarose gel was catalyzed by T4 DNA ligase (New England Biolabs) in T4 Buffer. The ratio of the insert DNA to the vector DNA added in the reaction mixture is about 10:1 (wt/wt) in order to optimize the ligation efficiency. This mixture was incubated at 16°C overnight to connect the adhesive ends of the insert and vector DNA. Self-ligation and inverted ligation can be largely limited in our reaction system because the two cohesive ends of the linear DNA are distinct. The ligation efficiency of identical cohesive ends (Nhe I to Nhe I or Sma I to Sma I) is dramatically higher than that of distinct cohesive ends (Nhe I to Sma I) (Sambrook & Russell, 2001).

5.2.5 Amplification and screening of the ligation products by transformation into bacteria

To amplify and also for screening of the correctly connected DNA, the ligation products were transformed into bacteria cells, particularly, DH5 α line of *Escherichia coli*. Firstly, DH5 α bacteria cells were made competent for DNA transformation in sterile. In brief, the DH5 α bacteria stock (a gift from Dr. Wang, A-Star, Singapore) was inoculated into 5 ml Lysogeny broth (LB) medium (consists of 1% tryptone, 0.5% yeast extract, 1% NaCl,

titrated to pH 7.0, autoclaved) and cultured at 37°C overnight, with vigorous shaking at 250 rpm. The second day, the 5 ml overnight culture was transferred into 200 ml antibiotics-free LB medium, and incubated at 37°C, 250 rpm for ~2.5 hr, until $OD_{600} = 0.5 \pm 0.05$, followed by centrifuge at 6000 rpm, 4°C, for 10 min. Then cells were resuspended in 100 ml of 0.1 M $MgCl_2$ by vortexing and incubated on ice for 5min. After incubation, cells were centrifuged again at 4500 rpm and 4°C for 10min, and resuspended in 20 ml of 0.1 M $CaCl_2$, and incubated at 4°C overnight. In the third day, cells were harvested by spinning at 4000 rpm and 4°C for 10min and resuspended on ice in 10 ml of sterile 86% $CaCl_2$ (w/v, 0.1 M), 14% glycerol (w/v). Finally, the resuspended cells were dispensed in 100 μ l aliquots and frozen in -80°C refrigerator for future use.

The DNA products after ligation were transformed into the competent cells using heat shock protocol. Briefly, 40 μ l of ligation product was mixed with 100 μ l competent cell stock and incubated for 30 min on ice, followed by a 2-minutes heat shock at 42°C, which permeabilizes the cell wall and the membrane of the competent cells to accept the plasmid DNA. Subsequently, cells were immediately cooled down to room temperature by incubation on ice. The transformed bacteria cells were then cultured in 500 μ l antibiotics-free LB medium at 37°C and 250 rpm for 60 min. The cell suspension was then spread onto LB plate (1.5% agar) with kanamycin and cultured at 37°C overnight.

As the successfully ligated DNA plasmids should possess resistance to kanamycin obtained from vector template, kanamycin would eliminate all the negative colonies. Positive colonies were then selected and inoculated into 5 ml fresh LB medium with

kanamycin to grow overnight at 37°C and 250rpm. The copy-number of the plasmid DNA increases as bacteria proliferate.

After overnight culturing, the plasmid DNA was harvested using the alkaline lysis method by a Quagen Mini-prep kit (Qiagen). The bacteria cells were centrifuged at 5000 rpm and 4°C for 10 min. First, bacteria cells were resuspended in the P1 resuspension buffer, which contains RNase to remove possible RNA contaminants. Then the cell wall and membrane were lysed by P2 buffer which contains detergent sodium dodecyl sulfate (SDS) for cell lysis and alkaline to denature proteins. After adding the acetate-containing N3 neutralization buffer, the proteins and chromosomal DNAs (large and less supercoiled) will precipitate, while small plasmid DNAs can renature and stay in the solution. The precipitations were removed by centrifuging at 13,000 rpm for 10 min. The suspensions containing the plasmid DNA were then loaded on resin columns for further purification. In brief, the DNA-loaded columns were washed by PB buffer to eliminate DNase which may degenerate the plasmids, and then washed by PE buffer in order to remove ions. Finally, the plasmids were dissolved in the elution buffer and collected in sterile tubes.

5.3 Verifying the constructed recombinant plasmids by gel electrophoresis and DNA sequencing

As described above, the (wt/dn)-(PKC α /PKC ϵ)-IRES-(EGFP/DsRed) plasmids were obtained by subcloning the PKC cDNAs into the pIRES2-EGFP or pIRES2-DsRed-Express vectors at the site of Nhe I and Sma I (Fig 5.3).

Fig. 5.3 Constructed recombinant plasmids of wt-PKC α -IRES-EGFP, dn-PKC α -IRES-EGFP, wt-PKC α -IRES-DsRed, dn-PKC α -IRES-DsRed, wt-PKC ϵ -IRES-EGFP, dn-PKC ϵ -IRES-EGFP, wt-PKC ϵ -IRES-DsRed and dn-PKC ϵ -IRES-DsRed.

In order to verify the engineered plasmids, gel electrophoresis was used to confirm the sizes of the harvested DNA plasmids. Figure 5.4 shows the DNA bands of the eight different cloning products, pre-digested by restrict endonuclease EcoR I or BamH I (New England Biolabs). These figures demonstrated that the sizes of all of the 8 recombinant plasmids were as expected (7000~7500 bp) and all of these DNA plasmids contain expected specific cloning sites (EcoR I or BamH I).

Fig. 5.4 Gel electrophoresis bands from the constructed recombinant plasmids. The plasmids of wt-PKC α -IRES-EGFP, dn-PKC α -IRES-EGFP, wt-PKC α -IRES-DsRed, dn-PKC α -IRES-DsRed, wt-PKC ϵ -IRES-EGFP, dn-PKC ϵ -IRES-EGFP, wt-PKC ϵ -IRES-DsRed and dn-PKC ϵ -IRES-DsRed were digested using EcoR I (A) or BamH I (B) before electrophoresis. The size of each DNA band was benchmarked by the GeneRuler DNA ladder marker and indicated in the figures.

As gel electrophoresis is unable to differentiate between the wild-type and dominant negative PKCs, DNA sequencing were performed (1st base, Singapore) in order to further confirm the identity of the plasmids. As anticipated, the constructed wt-PKC α -IRES-(EGFP/DsRed) plasmid exhibits 'AAG' while dn-PKC α -IRES-(EGFP/DsRed) demonstrated 'AAC' at base pair number 1102-1104 (Fig. 5.5A). And the dn-PKC ϵ -IRES-(EGFP/DsRed) shows 'TGG' instead of 'AAG' as found in wt-PKC ϵ -IRES-(EGFP/DsRed) at base pair 1309-1311 (Fig. 5.5B). These results proved that our recombinant plasmids contained the correct mutants of PKCs (wild-type or dominant negative).

Fig. 5.5 The result of DNA sequencing of the constructed recombinant plasmids. **A.** Sequence of nt. 1083-1123 in wild-type (top) and dominant negative (bottom) PKC α . The K368N mutation is achieved by mutating AAG to AAC (nt. 1102-1104) as labeled by the red rectangle. **B.** Sequence of nt. 1282-1322 in wild-type (top) and dominant negative (bottom) PKC ϵ . The K437W mutation is achieved by mutating AAG to TGG (nt. 1309-1311) as labeled by the red rectangle.

5.4 Transfection of the recombinant plasmids in PC12 cells

We examined the protein expression efficiency of these constructed plasmids in PC12 cells. The recombinant DNAs were introduced into PC12 cells using a non-viral method. Transfection was realized by ESCORT III transfection reagent (Sigma) which produces liposomes to encapsulate the DNA molecules inside, and in turn to fuse with the cell membrane and release the DNA into the cytosol. The expression efficiency was checked using an inverted fluorescent microscopy (Olympus Inc., Japan) 48 hours after transfection.

As shown in Fig. 5.6, all of the eight constructed recombinant plasmids can be transfected into PC12 cells. The fluorescent cell does not differ to non-fluorescent cells in morphology. However, the transfection rate was only 5~10%. The low efficiency of transfection is probably because the initiation of translation at IRES is not as efficient as at normal 5'-cap. Nonetheless, the transfection rate of 5~10% is sufficient for single cell recording. In a 12 mm coverslip, there are about $\sim 10^3$ fluorescent cells for recording.

In summary, we obtained recombinant plasmids containing genes of wt- or dn- form of PKC α or PKC ϵ and fluorescent report protein. The resulting plasmids are verified by gel electrophoresis and DNA sequencing. And transfection of the constructed plasmids into PC12 cells is feasible for single cell studies.

Fig. 5.6 Transfection of the recombinant plasmid or the vector plasmid in PC12 cells. The left column shows cell morphology viewed under bright field. The middle column shows the fluorescent transfected cells (excitation = 488 nm, emission = 507 nm for EGFP; excitation = 557 nm, emission = 579 nm for DsRed). The right column demonstrates the merged bright field image and the fluorescence image. In control experiments, the cells were transfected without plasmid, and no fluorescent signals were detected (top row). The scale bar represents 50 μ m.

CHAPTER 6

INVOLVEMENT OF PKC ALPHA IN PMA INDUCED FACILITATION OF EXOCYTOSIS AND VESICLE FUSION

6.1 Introduction

As described in Chapter 1, a large body of experiments has demonstrated that exocytosis is acutely regulated by protein phosphorylation (Jones & Persaud, 1998, Evans & Morgan, 2003), most notably via protein kinase C (PKC) (Morgan et al., 2005, Yang et al., 2002). Several key secretory proteins, such as SNAP-25 (Shimazaki et al., 1996, Nagy et al., 2002), synaptotagmin (Hilfiker et al., 1999, Haberman et al., 2005), Munc18 (Fujita et al., 1996, Barclay et al., 2003, Nili et al., 2006), and NSF (Matveeva et al., 2001) are PKC substrates, suggesting the versatile roles of PKC in various aspects of exocytosis. PKC functions are often examined using pharmacological tools such as PMA (PKC activator) and BIS (PKC inhibitor). In various cell systems, PMA has been shown to significantly potentiate exocytosis (Vitale et al., 1995, Pocotte et al., 1985, Gillis et al., 1996, Shoji-Kasai et al., 2002) and modulate vesicle fusion (Graham et al., 2000, Fulop & Smith, 2006). Many of these PMA effects are thought to be PKC dependent as they can be partially or completely eliminated by BIS treatment.

The exact mechanisms underlie PMA influences, however, often remain unclear largely because of the diversity of PKC isoforms and the caveats of pharmacological assessment

(reviewed in Chapter 1). To avoid these problems, overexpression of wild-type and dominant negative forms of individual PKC isoforms is instrumental to elucidate PKC-dependent PMA effects on exocytosis.

Among all PKC isoforms, PKC α is ubiquitously expressed PKC isoform found in all tissues, and is abundant in neuron and neuroendocrine cells. In particular, it is the only conventional PKC in chromaffin cells (Sena et al., 2001). In this chapter, we demonstrated that PMA facilitates exocytosis and fusion pore expansion mainly through PKC α in rat chromaffin cell derived PC12 cells, by overexpressing wild-type PKC α (wt-PKC α) and dominant negative PKC α (dn-PKC α) using the recombinant plasmids we obtained in the last chapter.

6.2 PMA modulates both the extent of vesicle exocytosis and the fusion kinetics

It has long been recognized that PMA, a stable analogue of an important signaling lipid diacylglycerol (DAG), enlarges the extent of Ca²⁺ dependent exocytosis in various cell types including PC12 cells and chromaffin cells (Graham et al., 2000, Barclay et al., 2003). Figure 6.1A presents typical amperometric recordings by carbon fiber microelectrodes in response to membrane depolarizing high K⁺ solution under different conditions. Each current spike corresponds to a vesicle fusion event. And the shape of each amperometric current spike unveils the kinetics of fusion pore opening and subsequent complete fusion or early closing. Confirming the results of Chapter 4, these

experiments showed that PMA significantly increased the number of exocytotic events per stimulus and this enhancement was completely eliminated by application of BIS. Furthermore, BIS alone inhibited exocytosis comparing to the control. Therefore, it is plausible to conclude that the PMA enhancement is PKC dependent and basal activities of PKCs is physiologically important to support exocytosis.

Also as noted from Fig. 6.1A, the amperometric spikes in the PMA treated cell generally exhibited larger amplitude comparing to that in the control cell. To examine this observation in details, amperometric spikes are extracted and analyzed individually on their sizes and shapes. The averaged amperometric spikes illustrated in Fig. 6.1B are obtained by averaging more than 400 recorded fusion events from more than 10 cells at different conditions. In contrast to that from the control cells, the averaged amperometric signal from the PMA treated cells manifests steeper rising phase indicating faster fusion pore opening and quicker releasing of catecholamines (Fig. 6.1B, left). As mentioned in Chapter 1, the rate of release or the rate of pore expansion can be quantified by the rise slope of the amperometric signal defined by a line-fitting between 35% and 90% of the peak amplitude (inset in Fig. 6.1C). As shown in Fig. 6.1C, the averaged rise slope in the PMA treated cells is significantly higher than that in the control cells (10.05 ± 0.29 pA/ms vs. 6.81 ± 0.20 pA/ms, $p < 0.001$). BIS was able to eliminate this PMA enhancement completely, implying the involvement of PKC activation (Fig. 6.1B, middle; and Fig. 6.1C). Application of BIS alone, however, did not cause apparent reduction in the fusion rate comparing to the control (Fig. 6.1B, right; Fig. 6.1C). Possibly, basal PKC activities are not required to support normal fusion pore opening. On the other hand, PKC

activation facilitates fusion probably by lowering the energy barrier of the fusion process. Similar PMA facilitation in fusion pore opening has also been found in chromaffin cells. In chromaffin cells, however, PMA at the same time causes early closing of fusion pore resulting in kiss-and-run type of fusion events of small quantal sizes due to partial release of catecholamines (Barclay et al., 2003, Graham et al., 2000).

In PC12 cells, the quantal size of vesicle release, or the total charge (Q) from integration of the amperometric current signal (Fig. 6.1D, inset), is also enlarged by PMA treatment. The distribution of Q is known to be severely skewed. Therefore, the cube root of spike charge ($Q^{1/3}$) is often used for analysis. $Q^{1/3}$ is shown to be proportional to the radius of the fusion vesicles, and has a Gaussian distribution (Clark & Ewing, 1997) so that Student's t -test can apply. As demonstrated in Fig. 6.1D, PMA treatment appreciably increased $Q^{1/3}$. The ability of BIS to reverse this PMA mediated enlargement (Fig. 6.1B, middle; Fig. 6.1D) again suggests the involvement of PKC. BIS application alone significantly decreased the quantal size (Fig. 6.1D), implying that basal PKC activities are important to maintain normal quantal size.

It is clear from our data as well as from others that PMA plays multiple regulatory roles in the Ca^{2+} dependent exocytotic process often through activation of PKCs. We further hypothesize that PKC α , which is the only abundant conventional PKC isoform in PC12 cells and whose activation depends on both Ca^{2+} and DAG/PMA, plays a dominant role in PMA mediated regulations.

Fig. 6.1 Effects of PMA and BIS on exocytosis of PC12 cells revealed by amperometry measurements. **A.** Representative amperometric recordings from a control, PMA treated, BIS and PMA treated and BIS only treated cells, in response to 60s stimulation of secretagogue high K^+ solution. **B.** Averaged amperometric spikes of all fusion events from 22 control cells, 11 PMA treated cells, 11 BIS+PMA treated cells or 19 BIS treated cells. **C.** Statistics of the spike rise slope. The rise slope is defined as the slope of the linear fit between 35% and 90% of the peak amplitude of the spike, as shown in the inset. Kolmogorov–Smirnov test: *** $p < 0.001$. **D.** Statistics of cube root of the spike charge ($Q^{1/3}$). The spike charge (Q) was defined as the integration of the amperometric spike current as shown in the inset. Student's t -test: ** $p < 0.01$, *** $p < 0.001$. For C and D, data are shown as mean $\pm$ SEM. The vertical and horizontal scale bars in the insets correspond to 5 pA and 5 ms, respectively.

6.3 Activation of PKC α underlies PMA induced enhancement in exocytosis

As described in the last chapter, wt-PKC α or dn-PKC α (created by a K368N mutation to permanently inactivate the ATP binding site of PKC α catalytic domain) are overexpressed in PC12 cells via transfection. cDNA of wt-PKC α or dn-PKC α is constructed together with cDNA of EGFP in the same plasmid *via* an IRES (Baird et al.), which links two open reading frames. Therefore, fluorescent EGFP proteins are co-expressed with PKC α molecules as reporter.

Fig. 6.2. PMA induced enhancement in exocytosis is PKC α dependent. Data was obtained by averaging the responses from 14 empty vector expressing cells (vector), 13 PMA treated vector cells (vector + PMA), 16 wild-type PKC α expressing cells (wt- α), 14 PMA treated wt cells (wt- α + PMA), 21 dominant negative PKC α expressing cells (dn- α) and 18 PMA treated dn- α cells (dn- α + PMA), during 60 s high K⁺ stimulation. **A.** The cumulative spike number is plotted over time. **B.** The total numbers of the resulting spikes are shown as mean $\pm$ SEM. Student's *t*-test: * $p < 0.05$, ** $p < 0.01$.

Figure 6.2A depicts the averaged time courses of the amperometric responses during 60 seconds of high K^+ stimulation from PC12 cells transfected with different plasmids and with or without PMA treatment. Only bright green cells, in which EGFP (then also PKC α) was abundantly expressed, were chosen for recording. All the amperometric responses can be fitted by a single exponential with a time constant of ~ 30 s. Comparing to chromaffin cells, exocytosis from PC12 cells is slow and is not biphasic resulting from fast release from the readily releasable vesicle pool and subsequent slower release from the reserve vesicle pool.

The statistics of the amperometric responses under different conditions is shown in Fig. 6.2B. Overexpression of dn-PKC α largely reduced the extent of exocytosis by 34% in comparison with the cells overexpressed with the empty vector (IRES-EGFP), indicating that competitive inhibition of endogenous PKC α activities impairs exocytosis. This is analogous to BIS effect (Fig. 6.1A). PMA application failed to rescue exocytosis in these dominant negative cells, strongly supporting the notion that PMA enhances exocytosis essentially through activation of PKC α . Overexpression of wt-PKC α , on the other hand, gave only marginal increase in exocytosis than the vector control. Obviously, the abundance of PKC α is not sufficient to significantly enhance exocytosis. But, PMA together with PKC α overexpression caused pronounced enhancement, in a greater extent comparing to its potentiation in the vector cells (35%, $p < 0.05$ vs. 26%, $p < 0.01$). These observations are consistent with the hypothesis that PKC α activation is responsible for PMA induced enhancement.

6.4 PMA increases the rate of fusion pore expansion and enlarges the fusion quantal size in PKC α dependent manner

Fig. 6.3 PMA modulates vesicle fusion *via* PKC α . Analyses are based on the same experiments in Fig. 6.2. **A**. The averaged amperometric spikes of all events from differently treated cells. **B**. The rise slopes are shown as mean $\pm$ SEM. Kolmogorov-Smirnov test: *** $p < 0.001$, Not Significant (N.S.) for $p > 0.05$. **C**. The cube roots of spike charges ($Q^{1/3}$) are shown as mean $\pm$ SEM. Student's t -test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The averaged amperometric spikes recorded from more than 12 cells transfected with different plasmids and with or without PMA treatment are depicted in the Fig. 6.3A. The statistics on the rise slope and the quantal size is presented in Fig. 6.3B and 6.3C, respectively. As expected, PMA speeded up the pore expansion as evident by the increase in the rise slope in the empty vector expressing cells. By contrast, such PMA-induced acceleration is absent in the cells expressed with dn-PKC α . In other words, just like BIS (Fig. 6.1), dn-PKC α is able to abolish the PMA effect in this regard. This observation strongly suggests that PKC α activation underlies the phenomenon. Also similar to the BIS effect presented in Fig 6.1, overexpression of dn-PKC α without PMA treatment did not affect the rise slope. Therefore, basal PKC α activities probably are not required or not significantly involved in normal fusion pore opening. On the other hand, overexpression of wt-PKC α renders even steeper rise in amperometric signal. It appears that increase in the endogenous PKC α activities is sufficient to gear up the fusion machinery via protein phosphorylation.

In the vector cells, as anticipated from the earlier experiments (Fig. 6.1), PMA enlarged the quantal size. Again analogous to the BIS action, overexpression of dn-PKC α eliminated this PMA induced augment. Hence, PKC α activation is likely responsible for this phenomenon. Without PMA, dn-PKC α overexpressed cells gave amperometric signals of notably smaller quantal size, similar to the case where BIS was applied alone to the control cells (Fig. 6.1). It implies that basal PKC α activities are important to maintain normal quantal size. Although inhibition of basal PKC α activities reduced the quantal size, overexpression of wt-PKC α itself was not sufficient to boost the quantal size. Albeit

that the importance of PKC α is highlighted by these experiments, we can not rule out the possibility that PMA may, concurrently, enlarge the quantal size via PKC α independent ways.

6.5 Foot probability is lowered by PMA through PKC activities

Fusion between vesicular membrane and plasma membrane can not occur spontaneously because two opposing lipid membranes must overcome substantial energy barriers in order to fuse. So, protein fusion machines are needed to lower these energy barriers and provide direct driving force to overcome them. Sometimes the driving force barely overwhelms the energy barriers leading to slow fusion pore opening characterized by the small pre-spike foot in the amperometric signal (Fig. 6.4, inset) (Chow et al., 1992).

PMA accelerates fusion pore expansion, likely by lowering certain energy barriers of the fusion process. In line with this notion, the probability to record a foot signal should also be reduced by PMA. As demonstrated in Fig. 6.4, it is indeed the case. Foot probability is obtained by calculating the percentage of the events that have a preceding foot among all the recorded exocytotic events which have amplitude greater than 20 pA. Foot can only be definitely resolved for events with relatively large amplitude (Wang et al., 2006). Not surprisingly, this PMA effect is also PKC dependent because BIS was able to inhibit it while BIS application alone increased the foot probability. PMA showed similar, but to a lesser extent, effects in the cells transfected with different plasmids. With reference to the vector expressing cells, overexpression of dn-PKC α behaved just like BIS to increase the

foot probability, whereas overexpression of wt-PKC α decreased the foot probability. These experiments demonstrate that PKC activities, including but maybe not exclusive to PKC α , lower the fusion energy barriers leading to decrease in the number of footed events.

Fig. 6.4 Effects of PMA, BIS and PKC α expression on foot probability. Representative amperometric spikes with and without a preceding foot are illustrated in the inset. Foot probability is defined as the percentage of footed signals among all the events that were obtained from the same experiments as in Fig. 6.1 and 6.2 and have amplitude greater than 20 pA (43 - 212 events).

6.6 Conclusion and discussion

PMA, which mimics the membrane lipid DAG to recruit conventional and novel PKCs as well as other secretion regulatory molecules such as Munc13 to the plasma membrane, is able to modulate exocytosis in various cells and in multiple ways. Herein, we report that

PMA increases the extent of exocytosis, facilitates vesicle fusion and enlarges quantal release in PC12 cells, largely through activation of PKC α .

Fig. 6.5 PMA induced enhancement in exocytosis is insensitive of 1-butanol treatment. Data were obtained by averaging the responses from 22 control cells, 11 PMA treated cell, 11 1-Butanol treated Cells (1-But) and 6 cells treated with both PMA and 1-butanol (PMA+1-But), during 60 s high K⁺ stimulation. **A.** The cumulative amperometric spike number is plotted over time. **B.** The averaged amperometric spikes of all events from differently treated cells. **C.** The rise slopes are shown as mean $\pm$ SEM. Kolmogorov–Smirnov test: *** $p < 0.001$ vs. control.

The observations that PKC α accelerates fusion pore opening and decreases foot probability, suggest that PKC α lowers the energy barriers in the fusion process. This can be achieved by modulating the fusion protein machinery or altering the lipidic structures of fusion pore through lipid enzymes. As a possible scenario, phospholipase D (PLD), which is reported to be activated by PKC (Hammond et al., 1997), produces phosphatidic

acids (PAs) at the inner leaflet of the cell membrane at the fusion site. In turn, the resulting cone-shaped PAs energetically favor the formation of negatively curved fusion pore (Vitale et al., 2001, Liscovitch et al., 2000, Humeau et al., 2001). To address this possibility, we investigated the effects of 1-butanol, which blocks formation of PA by competitive binding to PLD (Klein, 2005), on the fusion kinetics, and found that 1-butanol attenuated the spike slope from 6.81 ± 0.20 pA/ms (22 untreated cells) to 4.14 ± 0.35 pA/ms (11 treated cells) ($p < 0.001$) (Fig. 6.5). This result is agreeable with the previous finding using catalytically inactivated mutation (K898R) of PLD1 (Vitale et al., 2001). As shown in Fig. 6.5C, we further discovered that PMA rescued the rise slope of 1-butanol treated cells (6 cells) to a level even faster than the control (8.70 ± 0.85 pA/ms vs. 6.81 ± 0.20 pA/ms, $p < 0.001$). These preliminary experiments imply that it is unlikely that PMA (thus PKC) facilitates fusion pore expansion through activation of PLD.

PKC phosphorylates the machinery proteins (SNARE proteins, e.g. SNAP-25) and proteins binding to SNARE (e.g. Munc18). Botulinum neurotoxin E (BoNT/E) that cleaves SNAP-25 failed to modulate quantal fusion kinetics (Graham et al., 2000), suggesting that the SNAP-25 may not contribute to the dynamics of fusion pore. Hence, Munc18 is a more likely candidate that underlies PKC α induced fast pore expansion, as it has been shown to accelerate the spike rise in chromaffin cells depending on its phosphorylation at Ser313 and Ser306 by PKC (Craig et al., 2003, Barclay et al., 2003). Consistent with the observation in chromaffin cells, PKC α activities in PC12 cells also accelerate fusion pore expansion thus initial release. But on the other hand, PKC α

activation in PC12 cells, does not lead to early termination of pore fusion. In line with our observations, R39C mutation of Munc18, which loses the capability to bind syntaxin, was reported to reduce the quantal size in chromaffin cells (Fisher et al., 2001), but fail to alter the quantal size and kinetics in PC12 cells (Schutz et al., 2005). Therefore, it is likely that a different PKC α target regulates the pore closure in chromaffin cells.

Complete abolishment of PMA effects on total exocytosis per stimulation and fusion pore expansion rate in the cells expressed with dominant negative PKC α provides strong evidence that these PMA actions are largely relied on activation of PKC α . PKCs, which phosphorylate a spectrum of intracellular substrates on serine and threonine residues, are the major intracellular signal transduction proteins. Thus it is of no surprise to find that activation of PKCs constitutes a key event in upregulating secretory strength in neurons and endocrine cells at several stages of exocytotic cascade mediated by phosphorylation of distinct protein targets. Particularly, it is not surprising that PKC α , which is an abundantly expressed and most active conventional PKC isoform in PC12 cells (Wooten et al., 1992, Borgatti et al., 1996) and whose activation depends on both DAG and Ca²⁺, simultaneously implicates in multitude aspects of Ca²⁺-triggered exocytosis in PC12 cells as we show here. Synchronizing with Ca²⁺ triggering signal, PKC α may promote the efficiency of exocytosis synergistically with multiple Ca²⁺ activating steps or pathways.

We, nevertheless, cannot rule out the possible involvement of other PKC isoforms underlying PMA regulations, due in part to cross-reactivity in the enzyme-substrate binding among all the PKC isoforms. A novel PKC isoform PKC ϵ has been shown to

implicate in regulating activity-dependent potentiation of exocytosis through actin rearrangement in chromaffin cells (Park et al., 2006). Thus, we carried out experiments on cells overexpressed with wt- or dn-PKC ϵ . We found that, PKC ϵ plays distinct regulatory roles in contrast to PKC α . Specifically, PKC ϵ is not able to modulate the rate of fusion pore expansion (wt-PKC ϵ : 14.40 ± 0.60 pA/ms, 25 cells vs. dn-PKC ϵ : 12.63 ± 0.75 pA/ms, 28 cells). And on contrary to the effect of nt-PKC α , nt-PKC ϵ increased the quantal size ($Q^{1/3}$) (wt-PKC ϵ : 3.55 ± 0.02 fC $^{1/3}$ vs. dn-PKC ϵ : 3.74 ± 0.03 fC $^{1/3}$). Finally, comparing to dn-PKC α , dn-PKC ϵ showed much less inhibitory effect on exocytosis (12.9% reduction). And we also noticed a recent study (Staal et al., 2008) on rat chromaffin cells in which the authors, mainly based on pharmacological assessments, concluded that it is novel PKC θ but not any conventional PKC isoforms that is important in maintaining normal quantal size and extent of exocytosis. The discrepancy between their findings and ours may be attributable to the difference in the cell types used because PC12 cells do not express PKC θ (Borgatti et al., 1996). Moreover, the stimulation protocol used in that study is different from ours. We recorded the amperometric responses during a 60s high K $^{+}$ stimulation while they recorded the responses after a brief (3s) high K $^{+}$ stimulation. In addition, unlike that from chromaffin cells which typically have hundreds of releasable pre-docked vesicles, the amperometric responses from PC12 cells are essentially resulted from the release of newly arrived vesicles (Chapter 4). Nonetheless, to address the specific roles of other PKC isoforms present in PC12 cells (e.g. PKC β , δ and η) is still an intrusive and challenging topic in the future.

CHAPTER 7

PKC EPSILON FACILITATES RECOVERY OF EXOCYTOSIS IN PC12 CELLS

7.1 Introduction

The cascaded events in the exocytotic process are highly regulated by a plethora of secretor proteins, most notably SNAREs (reviewed in Chapter 1). It is thus not surprising that exocytosis is modulated acutely by protein phosphorylations catalyzed by various kinases, importantly, by protein kinase C (PKC), as described above. It has been well documented that PKCs play multifaceted roles in regulating exocytosis. But how a specific aspect of exocytosis is regulated by a specific PKC isoform has yet to be fully elucidated mainly because of the variety of PKC isoforms and the dubious specificity of the commonly used pharmacological agents. PKC activators such as PMA and inhibitors such as BIS non-selectively activate or inhibit multiple PKC isoforms. In addition, these pharmacological agents may exert nonspecific effects irrelevant to PKC pathways (Rhee et al., 2002, Alessi, 1997).

In this chapter, we overexpressed wild-type (wt-) or dominant negative (dt-) PKC ϵ in PC12 cells using the plasmids we constructed (Chapter 5). Based on the single-cell study platform mentioned in Chapter 1, we demonstrate that PKC ϵ facilitates recovery of the extent of exocytosis after an exhausting stimulation in a phosphatidylinositol-4,5-

bisphosphate (PIP₂) dependent manner. Furthermore, PKC ϵ mediates fast recovery of the vesicle fusion kinetics. This study provides evidence that PKC ϵ plays a regulatory role in shaping the plasticity of exocytosis.

7.2 Exocytosis in PC12 cells declines in response to a continuous stimulation and such exocytotic rundown takes time to recover

Exocytosis of synaptic vesicles at neuronal synapses or large dense core vesicles from endocrine cells declines overtime in response to repetitive or continuously maintained stimulation (Cousin & Robinson, 2000). Such exocytotic rundown is important to prevent unnecessarily (even harmfully) over-response to strong stimulations, and to avoid complete depletion of releasable vesicles available for following stimulations. The competence of exocytosis can be fully recovered when cells are allowed to rest for a sufficient period of time. The regulations on the exocytotic recovery shape the short-term plasticity of neurons (Zucker & Regehr, 2002).

Figure 7.1A presents a typical amperometric recording from a neuroendocrine PC12 cell when it was stimulated by local perfusion of high K⁺ solution which triggers exocytosis by causing membrane depolarization and subsequent activation of Ca²⁺ current. Each amperometric current spike, recorded by a carbon fiber microelectrode positioned on the cell surface, corresponds to an event of single vesicle release of catecholamine molecules. It is evident that the frequency of exocytotic events decreased gradually over time when the cell was challenged by a long high K⁺ stimulation (4 minutes).

Fig. 7.1 Rundown and recovery of exocytosis in PC12 cells. **A.** A typical amperometric recordings from a PC12 cell in response to a long high K^+ stimulation (4 minutes). **B.** The cumulative spike number during 4-minute stimulation averaged from 7 cells was plotted against time. **C and D.** Representative amperometric recordings from a PC12 cell in response to a paired 60s-high- K^+ stimulation separated by a recovery period of 30s and 120s, respectively. **E and F.** The average cumulative spike number over time resulting from the first and the second stimulation in cells challenged by the protocol presented in C (8 cells) and the protocol presented in D (8 cells), respectively. The horizontal dark lines in A, C and D indicate high K^+ stimulation.

The cumulative event number over time (Fig. 7.1B, average from 7 cells) can be fitted by an exponential with a time constant of ~60 seconds which indicates the time scale needed to deplete the releasable vesicles in a continuous stimulation. In the experiments demonstrated in Fig. 7.1C and D, two consecutive exhausting stimulations (each high K⁺ stimulation lasted for 60s) were delivered to a PC12 cell with a recovery period of 30s or 120s in between. The average responses to the protocol of 30s recovery and to the protocol of 120s recovery are shown in Fig. 7.1E and F. Clearly, exocytosis barely recovered 30s after the first intensive stimulation ($45.6 \pm 4.2\%$, 8 cells), while it largely restored with 120s' rest ($74.7 \pm 10.8\%$, 8 cells).

7.3 PKC ϵ facilitates the recovery of the frequency of exocytotic response

PKC ϵ is a novel PKC isoform expressed abundantly in PC12 cells (Wooten et al., 1992). It has been shown that it facilitates activity dependent potentiation in bovine chromaffin cells (Park et al., 2006) and synaptic potentiation in rat nerve terminal (Saitoh et al., 2001). We herein investigate the roles of PKC ϵ in recovering exocytosis. The dual stimulation protocol with either 30s recovery (Fig. 7.2A) or 120s recovery (Fig. 7.2B) was applied to the PC12 cells overexpressed with wt-PKC ϵ , dn-PKC ϵ or vector control.

Fig. 7.2 PKC ϵ facilitates the recovery of exocytosis. **A.** The cumulative amperometric responses to the dual high K⁺ stimulation with 30s recovery time in between (protocol shown in Fig. 7.1C). The average responses to the first stimulation from 14 vector overexpressing cells (gray), 12 wt-PKC ϵ overexpressing cells (dark) and 12 dn-PKC ϵ overexpressing cells (dashed) are shown on the left. The average responses to the second stimulation after 30s of recovery from the same groups of cells are shown on the right. **B.** The amperometric responses to the dual stimulation with 120s recovery (protocol shown in Fig. 7.1D), averaged from 12 vector cells, 12 wt-PKC ϵ cells, and 14 dn-PKC ϵ cells, respectively. **C.** The statistics of the recovery percentage (spike number obtained in the second stimulation divided by that from the first stimulation in the same cell) is shown as mean $\pm$ SEM, with the recovery period of 30s (from the cell groups used in **A**, and 11 cells transfected with siRNA against PKC ϵ) or recovery period of 120s (from the cell groups used in **B**, and 9 siRNA transfected cells). **D.** The statistics of the recovery percentage from 10 wt-PKC α and 13 dn-PKC α cells for 30 s recovery and 11 wt-PKC α and 7 dn-PKC α cells for 120 s recovery. Student's *t*-test: * $p < 0.05$, ** $p < 0.01$ vs. vector control.

Although the average exocytosis triggered by the first high K^+ stimulation in the cells overexpressed with wt-PKC ϵ appears to be slightly enhanced as compared to that from the vector cells (50.2 ± 4.2 vesicles averaged from 24 wt-cells vs. 43.1 ± 3.9 vesicles averaged from 26 cells, Fig. 7.2A and B), such increase is not statistically significant ($p > 0.05$). On the other hand, overexpression of dn-PKC ϵ led to minor inhibition 34.4 ± 4.0 vesicles, 26 dn-cells, $p < 0.05$). Although overexpression of wt-PKC ϵ did not enlarge exocytosis in the first stimulation, it led to stronger recovery in the second stimulation 30s later as compared to the recovery in the vector and dn-PKC ϵ overexpressing cells (Fig. 7.2A, right). Figure 7.2C (left) shows the statistics of the recovery percentage for these cells (12 wt-PKC ϵ cells: $77.2 \pm 12.6\%$; 12 dn-PKC ϵ cells: $39.4 \pm 6.6\%$, 14 vector cells: $46.2 \pm 5.8\%$). When the vector cells were allowed to rest for 120s, their exocytosis in the second stimulation was largely recovered (Fig. 7.2B and 2C: $84.9 \pm 9.3\%$, 12 cells) to an extent similar to the wt-PKC ϵ overexpressing cells ($86.3 \pm 9.3\%$, 12 cells). However, recovery of the dn-PKC ϵ overexpressing cells was still suppressed after 120s' rest ($60.8 \pm 7.2\%$, 14 cells). Knockdown of PKC ϵ by small interfering RNA (siRNA) transfection acted similarly as overexpression of dn-PKC ϵ , except that it reduced the efficiency of longer-term (120s) recovery to an extent ($44.6 \pm 6.5\%$, 9 cells) even greater than dn-PKC ϵ inhibition. The negative control of siRNA transfection did not show difference with the vector cells (data not shown). The inhibitory effects of dn-PKC ϵ and siRNA knockdown corroborate the importance of PKC ϵ in exocytotic recovery.

In contrast to PKC ϵ , PKC α did not show facilitating effect on exocytotic recovery. As shown in Fig. 7.2D, the recovery percentages at 30s in wt-PKC α overexpressing cells ($46.6 \pm 10.6\%$, 10 cells) and the dn-PKC α ($47.8 \pm 7.8\%$, 13 cells) are comparable to that in the vector cells. In addition, there is no significant difference in 120s recovery between the dn-PKC α overexpressing cells and the wt-PKC α overexpressing cells ($69.1 \pm 14.6\%$, 7 dn-PKC α cells vs. $64.9 \pm 14.2\%$, 7 wt-PKC α cells; $p > 0.05$). This suggests the regulatory role in exocytotic recovery is not universal to all PKC isoforms.

7.4 PKC ϵ mediated fast recovery is not due to enhanced Ca $^{2+}$ signal

There are evidences that PKCs down-regulate Ca $^{2+}$ current in some cells (Hu et al., 2000) and up-regulate it in other cell types (McMahon et al., 2000, Burgos et al., 2007, Doerner & Alger, 1992). We monitored the dynamics of the intracellular Ca $^{2+}$ concentration to examine whether PKC ϵ facilitates exocytotic recovery by enhancing Ca $^{2+}$ signal. The intracellular Ca $^{2+}$ concentration during the dual stimulation protocol was reported by ratiometric fluorescence measurement using a membrane-permeable Ca $^{2+}$ -sensitive dye (fura-2-AM). Figure 7.3A presents typical Ca $^{2+}$ responses to the dual high K $^{+}$ stimulation in vector overexpressing cells. As expected, the elicited Ca $^{2+}$ signal was transient even the stimulation persisted for long (1 minute) due to inactivation of voltage-activated Ca $^{2+}$ channels. The exocytotic rundown may be partly attributed to such attenuation of Ca $^{2+}$ signal (Forsythe et al., 1998, Borst & Sakmann, 1999). However, Ca $^{2+}$ current was able to recover largely from inactivation after 30s' rest (Fig. 7.3B: $88.7 \pm 3.8\%$ in terms of ratiometric measurement of the peak Ca $^{2+}$ concentration, 7 vector cells), in contrast to the

poor recovery of exocytosis within the same time frame (Fig. 7.2C). Neither overexpression of wt-PKC ϵ nor overexpression of dn-PKC ϵ affected Ca²⁺ response (both amplitude and time course) to the initial or the second stimulation (30s or 120s later). As demonstrated in Fig. 7.3B, The Ca²⁺ recovery in these cells is similar to that in vector cells (30s recovery: $93.1 \pm 6.7\%$ from 6 wt-PKC ϵ cells and $88.1 \pm 5.6\%$ from 9 dn-PKC ϵ cells; 120s recovery: $94.7 \pm 6.0\%$ from 5 wt-PKC ϵ cells and $100.4 \pm 3.3\%$ from 6 dn-PKC ϵ cells). Therefore, PKC ϵ regulates fast recovery of exocytosis through Ca²⁺ channel independent pathways.

Fig. 7.3 Dynamics of intracellular Ca²⁺ signal. **A.** Typical recording of intracellular Ca²⁺ concentration from a vector cell in response to the dual stimulation protocol with 30s (left) or 120s recovery time (right). The Ca²⁺ concentration is indicated by the ratio between the intensities of fluorescence emission when the Ca²⁺ sensitive dye (fura-2) is excited at 340 nm and 380 nm (F340/F380). The horizontal dark bars indicate the high K⁺ stimulation. **B.** The statistics on the recovery of the ratiometric measurements (Ca²⁺ signal) is depicted as mean $\pm$ SEM. The data on 30s recovery (left) is based on the recordings from 7 vector cells, 6 wt-PKC ϵ cells, and 9 dn-PKC ϵ cells. The data on 120s recovery (right) is based on the recordings from 6 vector cells, 5 wt-PKC ϵ cells, and 6 dn-PKC ϵ cells.

7.5 PKC ϵ mediated recovery is PIP2 dependent

PIP2 is a membrane phospholipid implicated in multiple aspects of exocytosis (Hay et al., 1995, James et al., 2008). For example, it plays a pivotal role in Ca²⁺ stimulated actin reorganization which is relevant to vesicle trafficking (Sheetz et al., 2006). And PIP2 may be involved in the PKC ϵ pathways via several mechanisms. Firstly, stimulated by membrane depolarization, PIP2 is converted to DAG by phospholipase C (PLC). And DAG subsequently activates PKC ϵ . In addition, it has been shown that PIP2 can interact directly with PKC ϵ (Shirai et al., 2007). Finally, some substrates of PKC ϵ , such as MARCKS, bind to PIP2 (Sheetz et al., 2006). We examined the roles of PIP2 in PKC ϵ enhanced exocytotic recovery using neomycin, a polycationic glycoside that selectively binds with membrane PIP2.

While sequestering of PIP2 by neomycin did not appreciably affect the extent of exocytosis responding to the first stimulation, it completely inhibited PKC ϵ mediated fast recovery (Fig. 7.4). The 30s recovery in the neomycin treated wt-PKC ϵ overexpressing cells ($n = 7$) was only $36.4 \pm 8.2\%$, comparable to that in the untreated vector cells. The neomycin inhibition on the PKC ϵ effect can be partially rescued by PMA (DAG analogue) to $50.1 \pm 7.4\%$ recovery (7 wt-PKC ϵ cells treated by both neomycin and PMA). This implies that PKC ϵ mediated fast recovery of exocytosis is achieved partly through PLC-PIP2-DAG-PKC ϵ pathway. On the other hand, both neomycin and PMA gave no obvious influences on dn-PKC ϵ overexpressing cells (Fig. 7.4).

Fig. 7.4 Neomycin impairs PKC ϵ mediated fast recovery of exocytosis (30s recovery). The statistics of the recovery percentage is shown as mean $\pm$ SEM. The data of untreated wt-PKC ϵ (column 1) or dn-PKC ϵ cells (column 4) is the same as in Fig. 7.2C. Recovery in wt-PKC ϵ cells ($n = 7$) is significantly inhibited after pre-treatment of neomycin (NEO) (column 2). Application of PMA to the same neomycin-pretreated wt-PKC ϵ cells partially rescued the neomycin inhibition (column 3). In contrast, neither neomycin pretreatment (column 5) nor subsequent PMA treatment (column 6) affected the recovery in dn-PKC ϵ cells ($n = 8$). The dash line represents the mean recovery percentage from untreated vector cells as shown in Fig. 7.2C. Student's t -test: * $p < 0.05$, ** $p < 0.01$.

7.6 PKC ϵ enhances the rate of vesicle delivery

As the number of pre-docked vesicles is limited (typically ~ 30 vesicles) and the carbon fiber can only record vesicular release from a small percentage of the total surface area of the cell ($\sim 5\%$) due to its small size, the amperometric events recorded by the carbon fiber in response to a long stimulation mainly comes from the new vesicles that arrived from the inner cytosol (Chapter 4). Therefore, it is plausible that PKC ϵ mediated fast recovery of exocytosis relies on enhanced rate of vesicle delivery to the plasma membrane.

Fig. 7.5 PKC ϵ mediates vesicle delivery from the inner cytosol to the subplasmalemmal region. The data is averaged from 5 vector cells (open circle), 8 wt-PKC ϵ cells (open square), 6 dn-PKC ϵ cells (filled square), 5 NEO-pretreated wt-PKC ϵ cells (cross), 5 wt-PKC α cells (open triangle), 5 dn-PKC α cells (filled triangle). **A.** The cumulative number of newly arrived vesicles to the subplasmalemmal region during 120s of TIRFM imaging without (left) and immediately after (right) 60s of high K^+ stimulation. The data was normalized by the value of vector control without high K^+ stimulation. **B.** The total number of visible subplasmalemmal vesicles at each snapshot during 120s of TIRFM recording without (left) and after (right) high K^+ stimulation. The total number of the subplasmalemmal vesicles remains stable (in equilibrium) as vesicle arrival and vesicle retrieval are balanced.

Individual fluorescently-labeled secretory vesicles near the plasma membrane can be resolved by total internal reflection fluorescence microscopy (TIRFM). We labeled the vesicles in PC12 cells by overexpressing NPY-EGFP and tracked individual vesicles in the subplasmalemmal region at 0.5s time interval using TIRFM. As revealed by TIRFM imaging, overexpression of both wt- and dn-PKC ϵ did not affect the average number of vesicles dwell in the subplasmalemmal region as compared to overexpression of vector control. The number of vesicles in the subplasmalemmal region remains stable as a result of the balance between the arrival of vesicles from the inner cytosol and the retrieval of vesicles away from the plasma membrane (Fig. 7.5B). The rate of vesicle trafficking was monitored. It was found that there is no difference in the rate of vesicle delivery between vector and dn-PKC ϵ cells (Fig. 7.5A). And in these cells, the delivery rates are similar before (Fig. 7.5A, left) and after one-minute high K⁺ stimulation (Fig. 7.5A, right). Overexpression of wt-PKC ϵ significantly enhanced the rate of vesicle arrival in the absence of stimulation (39.5% increase comparing with vector control, $p < 0.01$) (Fig. 7.5A, left). And in these wt-PKC ϵ cells, the delivery rate was further increased to 198.7% of control right after high K⁺ stimulation (Fig. 7.5A, right). The application of neomycin completely blocked the PKC ϵ induced acceleration in vesicle transport both before and after high K⁺ stimulation ($p > 0.05$ vs. vector control). In comparison to PKC ϵ , overexpression of wt-PKC α also increased the vesicle delivery to 125.6% of control before stimulation compared to overexpression of vector, whereas, the vesicle delivery (134.4% of control) was not further enhanced after stimulation (Fig. 7.5A). It appears that high K⁺ stimulation potentiates the effects of PKC ϵ , but not PKC α , in facilitating vesicle

trafficking. Overexpression of both dn-PKC ϵ and dn-PKC α inhibited the rate of vesicle delivery. And stimulation induced potentiation was absent in both dn cells (Fig. 7.5A). It is also evident that both PKC ϵ and PKC α do not have obvious influences on the number of vesicles “docked” or “tethered” on the cell membrane (Fig. 7.5B) despite of their facilitating roles in vesicle trafficking.

Fig. 7.6 PKC ϵ mediate F-actin reorganization in a PIP2 dependent manner. **A.** Representative confocal images of cortical F-actin network stained by Alexa633 conjugated phalloidin in a PC12 cell without stimulation (left) and a PC12 cell right after (right) 60s of high K⁺ stimulation. The scale bar = 5 μ m. **B.** The percentage of cells that have fragmented cortical actin network (as shown on right in **A**) under different conditions. Each data represented by mean $\pm$ SEM was from > 60 cells in 3 independent experiments. Student's *t*-test: * $p < 0.05$, ** $p < 0.01$ (reference is indicated by brackets); # $p < 0.05$ (reference to control after high K⁺, column 2).

7.7 PKC ϵ facilitates actin reorganization

The vesicles arrived from the inner cytosol have to overcome the physical barrier imposed by the dense actin meshworks underneath the plasma membrane in order to dock on the plasma membrane and prime into the readily releasable state (Trifaro et al., 2002). Upon Ca^{2+} triggering, the cortical actin network is disassembled and reorganized to clear the way for the newly arrived vesicles to reach the fusion sites. PKCs are known to promote actin reorganization (Rose et al., 2001). PKC ϵ , but not PKC α , was found to be recruited to the vesicle membrane upon Ca^{2+} stimulation (Mendez et al., 2003). Thus it may be the key PKC isoform to mediate the interactions between the vesicle and the actin network. In line with this notion, PKC ϵ has been shown to regulate the activity-dependent potentiation of exocytosis via actin rearrangement (Park et al., 2006).

Cortical actin network was fluorescently stained and examined by confocal imaging in the wt- or dn-PKC ϵ overexpressing PC12 cells without or right after one-minute high K^+ stimulation. It is evident from Fig. 7.6A that cortical actin network is usually dense and continuous at rest (left) and becomes thinner and discontinuous (fragmentation) after high K^+ stimulation (right). The percentage of cells which manifest fragmented actin cortex dramatically increased (from $15.8 \pm 3.5\%$ averaged from 234 control cells to $54.0 \pm 7.3\%$ averaged from 244 control cells) after high K^+ stimulation (Fig. 7.6B) because Ca^{2+} influx that accompanies membrane depolarization induces actin disassembly (Vitale et al., 1991, Neely & Gesemann, 1994). The observation that over-expression of dn-PKC ϵ greatly inhibited the post-depolarization disassembly of cortical actin (Fig. 7.6B, $30.8 \pm$

5.5% fragmentation, 98 cells) is consistent with the previous report in which membrane depolarization is induced by nicotine in mouse chromaffin cells (Park et al., 2006). On the other hand, overexpression of wt-PKC ϵ enhanced actin disassembly after high K⁺ stimulation ($79.9 \pm 4.9\%$ fragmentation, 67 cells). Such enhancement was blocked by neomycin ($32.9 \pm 10.6\%$ fragmentation, 64 cells), suggesting the critical involvement of PIP2. In comparison, the increase of actin fragmentation after high K⁺ stimulation in the wt-PKC α overexpressing cells was less prominent ($37.2 \pm 7.2\%$ from 94 cells without high K⁺ stimulation vs. $54.7 \pm 2.7\%$ from 94 cells after high K⁺ stimulation, Fig. 7.6B). It is likely that PKC ϵ regulation on actin dynamics underlies the PKC ϵ mediated fast vesicle delivery and fast recovery of exocytosis.

7.8 PKC ϵ facilitates the recovery of fusion kinetics

It is notable from Fig. 7.1A and C that a strong stimulation not only causes rundown in the frequency of exocytotic events, but also causes general decrease in the amplitude of the amperometric spikes indicating rundown in quantal vesicle fusion. To investigate this phenomenon, the amperometric signals were individually analyzed to reveal statistically the kinetics of vesicle fusion. From the control experiments on the vector cells, it was found that the average amplitude of the amperometric responses ($I_{\max}$) to the second stimulation (30s later) was largely reduced from 17.48 ± 0.56 pA to 12.2 ± 0.92 pA (14 cells, Fig. 7.7A). Longer recovery period (120s) did not help to restore the amperometric amplitude (Fig. 7.7A: 11.18 ± 0.57 pA, 12 cells) despite that the extent of exocytosis was regained to ~84.9% of the original competence (Fig. 7.2C).

Fig. 7.7 PKC ϵ facilitates the recovery of fusion kinetics. Analyses are based on the same data set presented in Fig. 7.2. **A-D.** The statistics of amplitude ($I_{\max}$), rise time (t_{rise}), half-width time ($t_{1/2}$) and charge (Q) of amperometric spike obtained from vector cells in response to the first stimulation and second stimulation after 30s or 120s recovery is shown as mean $\pm$ SEM. $I_{\max}$ is the peak amplitude of the amperometric signal (a, inset). t_{rise} is defined as the time duration as the amperometric signal rises from 35% to 90% of the peak amplitude (b, inset). $t_{1/2}$ is defined as the time duration as the amperometric spike rises to and then falls to 50% of $I_{\max}$ (c, inset). Q is the charge integration of the amperometric current (d, inset). Kolmogorov-Smirnov test with reference to the first stimulation: ** $p < 0.01$, *** $p < 0.001$. **E and F.** The ratios of $I_{\max}$, t_{rise} and $t_{1/2}$ (mean value obtained from the second stimulation divided by that from the first stimulation) are shown as mean $\pm$ SEM, with the recovery period of 30s (e) or 120s (f). Kolmogorov-Smirnov test with reference to the vector control: * $p < 0.05$, ** $p < 0.01$. **G.** Averaged amperometric spikes of all fusion events from vector (gray), wt- (solid), and dn- (dashed) PKC ϵ (top row) or PKC α (bottom row) cells, in response to the first stimulation (left: 1121 spikes from 26 vector cells; left-top: 1205 spikes from 24 wt-PKC ϵ cells, 894 spikes from 26 dn-PKC ϵ cells; left-bottom: 460 spikes from 17 wt-PKC α cells, 365 spikes from 20 dn-PKC α cells), and response to the second stimulation after 30s of recovery (middle: 289 spikes from 14 vector cells; middle-top: 435 spikes from 12 wt-PKC ϵ cells, 152 spikes from 12 dn-PKC ϵ cells; middle-bottom: 133 spikes from 10 wt-PKC α cells, 104 spikes from 13 dn-PKC α cells) or after 120s of recovery (right: 389 spikes from 12 vector cells; right-top: 489 spikes from 12 wt-PKC ϵ cells, 254 spikes from 14 dn-PKC ϵ cells; right-bottom: 68 spikes from 7 wt-PKC α cells, 100 spikes from 7 dn-PKC α cells).

The time it takes for the amperometric signal to rise from 35% to 90% of the peak amplitude (t_{rise}) reflects how fast the initial fusion pore formed between the vesicle membrane and the plasma membrane expands to discharge the vesicular cargo. The average t_{rise} of the amperometric signals elicited by the second stimulation (after 30s recovery) was apparently elongated as compared to that resulting from the first stimulation (Fig. 7.7B: 1.91 ± 0.18 ms vs. 1.32 ± 0.06 ms, 14 vector cells), indicating the slowdown of the fusion pore expansion. Such slowdown was restored only partially to 1.60 ms (± 0.07 ms) when the cells ($n = 12$) rested for 120s before the second stimulation. The time of the fusion process can be characterized by the half-width time of the

amperometric spike ($t_{1/2}$). As demonstrated in Fig. 7.7C, $t_{1/2}$ increased significantly after the first exhausting stimulation despite of 30s recovery (3.88 ± 0.17 vs. 2.63 ± 0.05 ms, $p < 0.001$) or 120s recovery (3.86 ± 0.12 ms). In contrast to the slowed fusion kinetics, the total charge of the amperometric signal (Q), which indicates the size of the vesicle (or total number of catecholamine molecules in the vesicle), did not change after the first strong stimulation (Fig. 7.7D). All these observations suggest that rundown in fusion kinetics (or fusion efficiency) occurs.

Investigation on the recovery of fusion kinetics was similarly carried out on the cells overexpressed with wt-PKC ϵ or dn-PKC ϵ . Overexpression of wt-PKC ϵ (12 cells) enabled almost full recovery in the fusion kinetics in 30s ($I_{\max} = 17.45 \pm 1.11$ pA, $t_{\text{rise}} = 1.40 \pm 0.06$ ms and $t_{1/2} = 2.63 \pm 0.09$ ms). It is also evident from the ratios of $I_{\max}$, t_{rise} and $t_{1/2}$ between the average from the second response and that from the first response (Fig. 7.7E), i.e., they are closer to 1 as compared to those from the vector control cells. As anticipated, overexpression of dn-PKC ϵ showed inhibitory effects in restoring the fusion efficiency. In this case (12 cells), the ratios of $I_{\max}$, t_{rise} and $t_{1/2}$ are $51.8 \pm 5.2\%$, $155.4 \pm 13.7\%$ and $169.3 \pm 9.0\%$, respectively. siRNA knockdown is similarly inhibitory (Fig. 7.7E). All these observations suggest the importance of PKC ϵ in recovery of vesicle fusion.

Intriguingly, the PKC ϵ facilitation on fusion recovery appears to be short-term because overexpression of wt-PKC ϵ did not make significant difference after 120s' rest as compared to the vector control (Fig. 7.7F). The average amperometric spikes responding to the first or the second stimulation (30s or 120s later) from the wt-PKC ϵ cells and the

dn-PKC ϵ cells are displayed in pair in Fig. 7.7G. The difference after 30s recovery is pronounced (Fig. 7.7G, middle), as compared to the small differences from the first stimulation (left) and after 120s recovery (right). Comparing to the recovery after 30s in the vector cells, the recovery in the wt-PKC ϵ overexpressing cells was much better while that in the dn-PKC ϵ overexpressing cells was significantly worse. These observations clearly demonstrate the facilitating role of PKC ϵ in fast recovery of quantal release. In contrast to PKC ϵ , PKC α plays a significant role in supporting the amperometric response to the first stimulation. As shown in Fig. 7.7G, in response to the first stimulation, overexpression of wt-PKC α (17 cells) significantly enhanced the $I_{\max}$ (to 121.4 ± 6.0 % of vector control, $p < 0.01$) whereas overexpression of dn-PKC α (20 cells) significantly inhibited the $I_{\max}$ (to 61.1 ± 4.1 % of vector control, $p < 0.001$). On the other hand, overexpression of wt-PKC α did not give better recovery compared to the control or overexpression of dn-PKC α . These observations demonstrate the distinct roles of PKC ϵ and PKC α in regulating the process of quantal vesicular release.

7.9 Conclusion and discussion

Multiple PKC isoforms have been identified in neuroendocrine PC12 cells. Among them, PKC ϵ is expressed most abundantly and PKC α is most active (Wooten et al., 1992). Based on overexpression of wt-PKC ϵ or its dominant negative, we herein provide evidences that PKC ϵ , but not PKC α , facilitates the recovery of exocytotic rate and kinetics of quantal vesicle fusion after an exhausting stimulation. Rundown in exocytosis after a strong stimulation may be an important built-in mechanism to prevent oversecretion. On

the other hand, timely recovery is important to ensure responsivity to the next stimulation. Therefore, a delicate balance shall exist between rundown and recovery through certain regulatory mechanisms. Our experiments postulate a PKC-dependent mechanism which may be relevant to the plasticity of exocytosis in both neuroendocrine and neuron cells.

We show that long-lasting stimulation to PC12 cells causes rundown not only in the frequency of exocytotic events but also in the fusion kinetics (specifically, smaller amperometric amplitude, slower fusion pore expansion and longer fusion time). As discussed earlier, the amperometric signals recorded by carbon fiber are mainly resulting from the release of newly arrived vesicles. And the rundown in the extent of exocytosis is not because of decline in the rate of vesicle arrival in presence of lasting stimulation (data not shown). Therefore, decrease in exocytosis is attributable to the lower release probability of the vesicles that reach the plasma membrane, probably as a consequence of depletion of readily releasable vesicles (Burrone & Lagnado, 2000, Moser & Beutner, 2000) or/and inactivation of Ca^{2+} current (Forsythe et al., 1998, Borst & Sakmann, 1999). As fusion kinetics has been found to be Ca^{2+} concentration dependent (Wang et al., 2006), slowdown in fusion kinetics may be due in part to Ca^{2+} inactivation as well. But, as demonstrated in Fig. 7.3, recovery of Ca^{2+} current is not the bottleneck to the recovery of exocytosis and fusion kinetics because Ca^{2+} current quickly restores while exocytosis recovers poorly within the same time frame (Fig. 7.2C and 7.7E). Therefore, the limiting steps for the exocytotic recovery may instead be vesicle docking, priming to releasable state, and additional gear-up of the fusion machinery to enhance fusion efficiency. It has been demonstrated that SNARE complex is the minimal fusion machinery that is able to

drive spontaneous fusion between artificial lipid vesicles but with very slow fusion kinetics (Weber et al., 1998). This is in line with the notion that fusion machinery underlying the normal vesicular fusion in cells acquires further gear-up in addition to complexing between vesicular and target SNARE proteins which brings the vesicular membrane and the plasma membrane in close contact. The recovery of exocytotic frequency and fusion kinetics is not synchronic. Specifically, in control cells, the extent of exocytosis restores to ~80% in 120s while fusion kinetics remains slow. It may imply the mechanistic difference in these two recovery processes. Further experiments are however needed to address this question.

Wt-PKC ϵ overexpressing cells only needs ~30s to restore to ~80% of the normal exocytotic competence after an exhausting stimulation, while 30s recovery of the dn-PKC ϵ overexpressing cells is only ~39%, worse than the vector control (~46%). PIP2-sequestering neomycin can completely eliminate PKC ϵ mediated fast recovery, suggesting the crucial involvement of PIP2. Based on these observations, the following scenario may be speculated. Membrane depolarization and/or Ca²⁺ influx activates PLC (Thore et al., 2004) which hydrolyzes PIP2 into DAG. In turn, DAG recruits and activates PKC ϵ . PKC ϵ activation then increases the release probability of the vesicle through phosphorylation of the secretory proteins relevant to vesicle docking, priming and fusion, such as SNAP-25 (Shimazaki et al., 1996, Nagy et al., 2002), synaptotagmin (Hilfiker et al., 1999, Haberman et al., 2005), and Munc18 (Fujita et al., 1996, Barclay et al., 2003, Nili et al., 2006). At the same time, Ca²⁺ influx together with PIP2 elicits actin

reorganization, clearing the way for vesicles from the large reserve pool in the inner cytosol to reach the plasma membrane. And this can be facilitated by PKC ϵ (Akita, 2008).

Since fusion proteins such as SNAP-25 are PKC ϵ targets, it should not be unexpected that PKC ϵ facilitates the recovery of fusion kinetics. However, such PKC ϵ facilitation appears to be short-term. As demonstrated in Fig. 7.7, in wt-PKC ϵ overexpressing cells, fusion kinetics recovered mostly in 30s whereas PKC ϵ facilitation disappeared after 120s' rest. This peculiar transient facilitation is similar to post-tetanic potentiation that occurs in neuron after intense stimulation as a form of short-term synaptic plasticity to increase the efficiency of synaptic transmission (Zucker & Regehr, 2002). It has been shown that post-tetanic potentiation critically relies on enhanced Ca²⁺ sensitivity of vesicle fusion mediated by PKC (Brager et al., 2003). In addition, PKC ϵ regulated actin reorganization may help to recover the fusion kinetics as it has been shown that actin reorganization may provide additional driving force for vesicle fusion (Eitzen, 2003, Berberian et al., 2009). We tested the effect of neomycin on PKC ϵ mediated recovery of fusion kinetics (data not shown). Although overexpression of PKC ϵ showed no facilitation under neomycin treatment, unequivocal conclusion cannot be drawn because neomycin treatment itself significantly impairs the fusion kinetics (smaller amperometric amplitude, longer t_{rise} and $t_{1/2}$).

PKC ϵ and PKC α differentially regulate exocytosis in PC12 cells. Compared to the vector control, overexpression of both wt-PKC ϵ and wt-PKC α did not significantly augment the extent of exocytosis responding to the first (single) stimulation, whereas overexpression

of dn-PKC α gave more severe inhibition compared to dn-PKC ϵ (21 dn-PKC α cells: $66.3 \pm 8.0\%$ of the control response, $p < 0.01$ vs. 26 dn-PKC ϵ cells: $79.8 \pm 9.2\%$, $p < 0.05$). Overexpression of both wt-PKC ϵ and wt-PKC α increased the amperometric amplitude. But the enhancement by PKC α was more prominent (17 wt-PKC α cells: $121.4 \pm 6.0\%$ of the control response, $p < 0.01$ vs. 24 wt-PKC ϵ cells: $112.1 \pm 4.2\%$, $p < 0.05$). These observations suggest the facilitating roles of PKC α on the exocytosis triggered by initial stimulation are more prominent compared to that of PKC ϵ . However, PKC α facilitations disappeared in the exocytotic response to the second stimulation (not significantly different from control). On the other hand, wt-PKC ϵ gave significant enhancement on exocytotic response to the second stimulation (30s after the first). Therefore, it is PKC ϵ , but not PKC α , that facilitates the fast recovery of the extent of exocytosis and fusion kinetics. Activation of PKC α is Ca²⁺ dependent. The actions of PKC α seem to synchronize with the initial stimulation (and accompanying Ca²⁺ signal) while the actions of PKC ϵ , whose activation does not require Ca²⁺, seem to be delayed. And effects of both PKC α and PKC ϵ are transient. The distinct roles of PKC isoforms may be possibly due to the timing of their activation. However, the underlining mechanisms still require further studies to elucidate.

CHAPTER 8

GENERAL CONCLUSIONS AND PERSPECTIVES

8.1 PKC plays multifaceted roles in exocytosis as revealed by single cell studies

PKC has long been identified as a key regulator of exocytosis. However, due to the complexity of the exocytotic cascade (reviewed in Chapter 1), the specific regulatory roles of PKC in exocytotic pathways are still poorly understood. Our single cell study platform combining molecular biology, bio-imaging, and electrochemical approaches enable us to examine a particular aspect of the dynamic exocytotic process at single cell level with high temporal resolution and high sensitivity (even single vesicle sensitivity). We found that PKC modulates the extent of exocytosis, vesicle delivery rate, vesicle lateral movement, vesicle fusion kinetics (rate and duration of fusion pore opening, quantal size, amplitude, pre-spike foot), actin reorganization, and recovery of exocytosis and fusion kinetics after an exhausting stimulation.

Activation of PKC via PMA treatment increases the frequency of amperometric spikes from PC12 cells in response to high K^+ depolarization whereas inhibition of PKC by BIS reduces it. This is consistent with observations on chromaffin cells (Vitale et al., 1995, Gillis et al., 1996), beta cells (Wan et al., 2004) and neurons (Stevens & Sullivan, 1998, Soliakov & Wonnacott, 2001). Overexpression of dn-PKC α and dn-PKC ϵ also impairs

exocytosis, corroborating the essential roles of PKC in supporting exocytosis. PKC enlarges the extent of exocytosis mainly through increasing the delivery rate of vesicles from inner cytosol to the plasma membrane instead of increasing the total number of vesicles in the subplasmalemmal region as revealed by TIRFM imaging. And confocal imaging showed that enhanced vesicle transport may be resulted from PKC mediated F-actin reorganization. It was also found that vesicles in the subplasmalemmal regions can be differentiated into three distinct populations differing in their motion characteristics and fusion competence, and PKC activation by PMA modulates these vesicle populations differently. Furthermore, we provide evidence that PMA treatment lowers the energy barrier of membrane fusion, increases the rate of fusion pore expansion and the quantal size of single fusion events, likely through activation of PKC α . Synaptic neurons or neuroendocrine cells undergo reversible rundown in exocytosis in response to a persistent stimulation. Recovery of exocytosis after an exhausting depolarization characterizes the ability of cells to regain the competence of exocytosis. We found that PKC ϵ , but not PKC α , facilitates the recovery of exocytosis after exhausting stimulation in a PIP2 dependent manner. It postulates a regulatory role of PKC in shaping the exocytotic plasticity that is relevant to short-term memory in the central nervous system. Taken together, our results highlight the multifaceted roles of PKC in facilitating different aspects of exocytosis.

8.2 PKC alpha and epsilon regulate exocytosis differentially

We examined the isoform-specific effects of PKCs, specifically, alpha and epsilon isoforms, which respectively are the most active and most abundant PKC isoforms in

PC12 cells (Wooten et al., 1992). We found that PKC α and PKC ϵ play common and distinct roles in regulating different aspects of exocytosis.

To compare the roles of PKC α and PKC ϵ , table 8.1 summaries the statistic significance between the wild-type and the dominant negative form of PKC α or PKC ϵ in different exocytotic aspects. It appears that the effects of PKC α is more prominent in exocytosis triggered by the first (or single) stimulation, in terms of enhancing the extent of exocytosis, the amperometric amplitude, the quantal size and the rise slope. In contrast, the effects of PKC ϵ in exocytosis responding to the second stimulation after a short-period of recovery from an exhausting stimulation are more significant comparing to that of PKC α , in terms of increasing the extent of exocytosis and $I_{\max}$ and vesicle delivery and actin reorganization, and decreasing amperometric half-width time and time constants of decaying phase of the amperometric signal (tau1 and tau2) and quantal size and rise time. In general, it appears that PKC α makes the initial exocytosis more efficient whereas PKC ϵ facilitates the recovery of exocytotic efficiency.

In summary, our findings, together with several previous studies (Chapter 1) start to reveal the isoform-specificity of PKC regulations. Given the importance, diversity and complexity of PKC regulations in exocytosis, elucidating specific roles of individual PKC isoforms is certainly, and still, a major critical challenge in the future.

Table 8.1 Different effects of PKC α and ϵ on different aspects**First (or single) stimulation**

	Spike number	$t_{1/2}$	$I_{\max}$	$Q_{1/3}$	t_{rise}	Rise slope	Fall tau1	Fall tau2
wt-PKC α								
vs.	++	--	+++	+++	-	+++	N.S.	-
dn-PKC α								
wt-PKC ϵ								
vs.	+	--	+	---	N. S.	N.S.	--	-
dn-PKC ϵ								

Second stimulation after 30 s recovery

	Spike number	$t_{1/2}$	$I_{\max}$	$Q_{1/3}$	t_{rise}	Rise slope	Fall tau1	Fall tau2
wt-PKC α								
vs.	++	N.S.	+	+++	N. S.	N. S.	+	N. S.
dn-PKC α								
wt-PKC ϵ								
vs.	+++	--	+++	---	--	+++	--	--
dn-PKC ϵ								

	Vesicle delivery before high K ⁺	Vesicle delivery after high K ⁺	Actin disassembly before high K ⁺	Actin disassembly after high K ⁺
wt-PKC α				
vs.	++	++	+	N.S.
dn-PKC α				
wt-PKC ϵ				
vs.	++	+++	N.S.	++
dn-PKC ϵ				

+ the value of wt is greater than that of dn

- the value of wt is smaller than that of dn

Student's *t*-test: (+, -) $p < 0.05$, (+ +, - -) $p < 0.01$, (+ + +, - - -) $p < 0.001$, N.S. $p > 0.05$ wt vs. dn.

8.3 Future works

The work presented here only uncovers a few interesting phenomenon related to PKC regulation which may provide insights to the highly complex process of exocytosis. Further investigates on the distinct regulatory roles of specific PKC isoform on specific aspects or molecule steps along the exocytotic cascade will remain as the major challenge in the future.

Our studies focused only on two PKC isoforms (PKC α and PKC ϵ). Nonetheless, PC12 cells also express other PKC isoforms including PKC β , δ and ζ (Wooten et al., 1992, Borgatti et al., 1996). It is thus of interest to investigate how other isoforms synergistically yet differentially regulate exocytosis. For example, PKC θ has been suggested to be critical in maintaining quantal vesicle in rat chromaffin cells (Staal et al., 2008). Does it do the same in PC12 cells? If so, how does PKC θ regulation on fusion kinetics differ from that of PKC α ? In another example, knockout of PKC δ impairs insulin secretion in pancreatic β cell, without reducing the number of docked vesicles (Uchida et al., 2007). Then how does PKC δ regulate the secretion of PC12 cell and its recovery? These questions need to be addressed in the future.

We aim to further reveal the underlying biomolecular mechanisms underlying the actions of PKC α and PKC ϵ in our future efforts. For example, does PKC α facilitate fusion pore opening by phosphorylating SNAP-25 or Munc18 or other unidentified PKC substrate? Some preliminary experiments have been carried out to explore the downstream

pathways of PKC. We found PMA induced PKC activation failed to increase the vesicle delivery rate in BDM (2,3-butanedione 2-monoxime), an inhibitor of myosin (Warren et al., 1985)) pretreated PC12 cells, suggesting the involvement of myosin in the PKC-mediated vesicle transportation. Moreover, the PMA-enhanced extent of exocytosis and expansion rate of the fusion pore were impaired by inhibiting the activity of Rho GTPase (using *Clostridium difficile* Toxin B as inhibitor (Just et al., 1995)) but not by competitive inhibition of PLD using 1-butanol (Klein et al., 2005). Hence, Rho GTPase but not PLD may be another possible target of PKC. PKC α and PKC ϵ seem to act in different timing with reference to the initial high K⁺ stimulation. What are the molecular reasons underlying? Are the differential actions of PKC α and PKC ϵ due to the differences in their location, phosphorylation targets, or activation mechanism? Obviously, tremendous effects are still needed to address these intriguing yet important questions.

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List of publications

- **Xue R.**, Zhao Y., Su L., Ye F. & Chen P. PKC epsilon facilitates recovery of exocytosis after an exhausting stimulation. *Pflugers Archiv – European Journal of Physiology* (Submitted).
- Zhang J.[§], **Xue R.**[§], Ong W. Y. & Chen P. Roles of cholesterol in vesicle fusion and motion. *Biophysical Journal* (under revision). [§] The authors have contributed equally to this work
- **Xue R.**, Zhao Y. & Chen P. 2009. Involvement of PKC alpha in PMA induced facilitation of exocytosis and vesicle fusion in PC12 cells. *Biochemical and Biophysical Research Communications* **380**:371-376.
- Béguin P., Nagashima K., Mahalakshmi R. N., Ng M. Y., Ng Y. J. A., Tay H. S., Hwang L. A., Firsov D., Tang B. L., Mori Y., Inagaki N., Seino S., **Xue R.**, Chen P. & Hunziker W.. VGCC-BARP: a novel regulator of voltage-gated Ca²⁺-channels and calcium-dependent exocytosis. *Neuron* (under revision).
- Huang Y., Sudibya H. G., Fu D., **Xue R.**, Dong X., Li L. J. & Chen P. 2009. Label-free detection of ATP release from living astrocytes with high temporal resolution using carbon nanotube network. *Biosensors & Bioelectronics* **24**:2716-2720.
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