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<td>Xue, Renhao; Zhao, Yanying; Su, Luanyu; Ye, Feng; Chen, Peng</td>
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PKC epsilon facilitates recovery of exocytosis after an exhausting stimulation

Renhao Xue, Yanying Zhao, Luanyu Su, Feng Ye, Peng Chen*

Division of Bioengineering, Nanyang Technological University, Singapore 637457

* Corresponding author: Fax: +65-67911761; E-mail: chenpeng@ntu.edu.sg
Abstract

It has been well documented that protein kinase Cs play multifaceted roles in regulating exocytosis of neurotransmitters and hormones. But the isoform-specific PKC effects are still poorly elucidated mainly because of the large variety of PKC isoforms and the dubious specificity of the commonly used pharmacological agents. In the present study, based on overexpression of wild type (wt) or dominant negative (dn) PKCε, we demonstrate in neuroendocrine PC12 cells that PKCε, but not PKCα, facilitates recovery of exocytosis after an exhausting stimulation. Specifically, PKCε mediates fast recovery of the extent of exocytosis in a phosphatidylinositol biphosphate (PIP2) dependent manner, likely through enhancing the rate of vesicle delivery and reorganization of cortical actin network. In addition, PKCε promotes fast recovery of vesicle release kinetics that is slowed after a strong stimulation. These experimental results may suggest a PKC dependent mechanism relevant to the short term plasticity of exocytosis in both neurons and neuroendocrine cells.

Keywords exocytosis • protein kinase C • exocytotic recovery • amperometry
Introduction

Vesicle exocytosis is a fundamental cellular process whereby signaling molecules such as neurotransmitters or hormones are discharged from cargo-laden secretory vesicles into the extracellular space upon vesicle fusion with the plasma membrane [10]. Before final Ca\(^{2+}\)-triggered vesicle fusion, secretory vesicles have to go through a sequence of steps involving approaching the plasma membrane, tethering and docking onto the plasma membrane, and priming to readily releasable state [43]. The cascaded events in the exocytotic process are highly regulated by a plethora of secretor proteins, most notably, soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) [42]. It is thus not surprising that exocytosis is modulated acutely by protein phosphorylation, e.g., mediated by protein kinase C (PKC) [29]. A number of key secretory proteins, such as SNAP-25 [32, 40], synaptotagmin [20], and Munc18 [5, 19], are PKC substrates, suggesting the versatile roles of PKC in various aspects of exocytosis.

PKC family consists of ten members [29], 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. It has been well-documented that PKCs play multifaceted roles in regulating exocytosis [29, 52-54]. But the isoform specificity of PKC effects is still poorly elucidated mainly because of the large variety of PKC isoforms and the dubious specificity of the commonly used pharmacological agents. PKC activators such as phorbol-12-myristate-13-acetate (PMA which is a stable DAG analogue) and inhibitors such as bisindolylmaleimide (BIS which abolishes PKC activity by preventing ATP binding with the PKC catalytic domain) non-selectively activate or inhibit multiple PKC isoforms. In addition, these pharmacological agents may exert nonspecific effects irrelevant to PKC pathways [2, 36].

In the present study, based on overexpression of wild-type (wt) or dominant negative (dn) PKCε, we demonstrate in neuroendocrine PC12 cells that PKCε facilitates recovery of the extent of exocytosis after an exhausting stimulation in a phosphatidylinositol-4,5-bisphosphate (PIP2) dependent manner. Furthermore, PKCε mediates fast recovery of the vesicle release kinetics. This study provides evidence that PKCε plays a regulatory role in shaping the plasticity of exocytosis.

Materials and Methods

Chemicals and solutions

The bath solution contains (in mM): 140 NaCl, 5.5 KCl, 2 MgCl\(_2\), 2 CaCl\(_2\), 10 glucose, and 10 HEPES (titrated to pH 7.4). High K\(^+\) solution for stimulation of exocytosis contains (in mM): 37 NaCl, 105 KCl, 5 CaCl\(_2\), 2 MgCl\(_2\), and 10 HEPES (titrated to pH 7.4). In some experiments, cells were incubated in the bath solution with addition of 100 µM neomycin for 30 minutes before experiment. For PMA treatment, 0.1 µM PMA was applied to the bath solution for 10 minutes. All chemicals were purchased from Sigma (St. Louis, MO, USA).

Cell culture

PC12 cells from American Type Culture Collection (MD, USA) were cultured in RPMI 1640 medium (Gibco, MD, USA) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Gibco), 5% heat-inactivated horse serum (Gibco), and 1% penicillin-streptomycin (Gibco) and maintained at 37°C in a humidified atmosphere of 5% CO\(_2\)/95% air. Prior to experiments, cells were treated with 5 µM dexamethasone for ~5 days as previous described [29, 45, 50] to enhance chromaffin cell phenotype and planted on poly-L-lysine (Sigma) pre-coated glass coverslips.
**Plasmids**

The cDNA plasmids coding for wt-PKCe, dn-PKCe (K437W mutated), wt-PKCa and dn-PKCa (K368N mutated) were obtained from the Addgene plasmid collection (Cambridge, MA, USA). The plasmid coding for enhanced green fluorescence protein (EGFP) conjugated neuropeptide-Y (EGPF-NPY) was a kind gift from Dr. Wolf Almers (Oregon Health Sciences University, USA). The pIRES2-EGFP and pIRES2-DsRed-Express vector was purchased from Clontech (Mountain View, CA, USA). The (wt/dn)-(PKCe/PKCa)-IRES-(EGFP/DsRed) plasmids were constructed by subcloning the corresponding cDNAs into pIRES2-EGFP or pIRES2-DsRed-Express vector at the sites of Nhe I and Sma I. These plasmids allow concurrent and separate expression of EGFP or DsRed with PKC to report transfection efficiency. PC12 cells were transfected with relevant plasmid 1-2 days prior to experiments using ESCORT-III transfection reagent (Sigma).

**RNA interference**

Small interfering RNA (siRNA) (1st Base, Singapore) targeting sequence 5’-atggtagtgttcaatggc-3’ (NM_017171.1, nt 194–211) or 5’-ttaaggagccacaagcagttatg-3′ (NM_001105713.1, nt 1572–1598) was used for knockdown of PKCe [24] or PKCa [26], respectively. Scrambled nontargeting siRNA was used as the negative control. The siRNA duplexes were conjugated with fluorescent reporter (Cy3). Transfection of siRNA was performed using DharmaFECT transfection reagent (Dharmacon, IL, USA), 2–3 days prior to experiments.

**Amperometry measurement**

Cells were stimulated by local perfusion of the high K\(^+\) solution through a glass micropipette (tip size of ~2 μm) positioned a few μm above. Amperometric signals were recorded at room temperature by a 5 μm-in-diameter carbon fiber microelectrode (ALA Scientific Instruments, NY, USA) biased at 700 mV, with a sampling rate of 4 kHz and low-pass filtering of 1 kHz using an EPC-10 double patch-clamp amplifier (HEKA Electronik, Lambrecht, Germany). The microelectrode was gently positioned onto the cell membrane to avoid diffusion-caused distortion in the signals. 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 release kinetics.

**[Ca\(^{2+}\)]\(_i\) imaging**

PC12 cells were loaded with 5 μM Fura2 acetoxymethyl ester (Fura2-AM, Molecular Probes, OR, USA) for 45 min in culture medium. The cells were then washed and rest in Fura2-AM free bath solution for at least 30 min before the experiment to allow hydrolysis of the ester thus retention of the dye. Fluorescent signals following the high K\(^+\) stimulation were recorded from single cells using a photometry system (TILL Photonics GmbH, Germany). The intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) were reported as the ratio of Fura2 fluorescence intensity resulting from excitation at 340 nm to that resulting from excitation at 380 nm (F340/F380).

**TIRFM imaging**

PC12 cells were co-transfected with NPY-EGFP to label the large dense core vesicles (LDCV) and PKC-IRES-DsRed plasmids. Individual fluorescent LDCVs were visualized using a Zeiss Axiovert 200 inverted microscope (Carl Zeiss Inc., Germany) equipped with a 100× TIRF (total internal reflection objective (1.45 NA). Time-lapse digital images were acquired, at 2 Hz, by a CCD camera controlled by MetaMorph 6.3 program (Molecular Devices, Downingtown, PA, USA).
Confocal imaging

PC12 cells, with or without one-minute high K⁺ stimulation, were fixed in phosphate buffer solution (PBS) (Gibco) containing 3.7% formaldehyde (Sigma) for 10 minutes followed by permeabilization. PC12 cells were permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 10 minutes and blocked with 1.5% bovine serum albumin (Sigma) for 1 hour at room temperature. After extensive washing by PBS, the cells were incubated in 1:1000 (v/v) of Alexa635 conjugated phalloidin (Molecular Probes) in PBS with 1.5% BSA for 40 min in 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).

Data Analysis

All the data analyses are performed using Igor pro 6.0.1 (WaveMetrics, OR, USA). The parameters of amperometric spikes were calculated using an Igor program, Amperometric Spike Analysis 8.15 [31]. Only spikes with amplitude > 2 pA (5 times of the background noise) were used for analysis. All the statistics is represented by mean ± SEM. Statistical significance is evaluated by paired or unpaired Student’s t-test, or Kolmogorov–Smirnov test for $Q$, $I_{\text{rise}}$, $I_{1/2}$, and $I_{\text{max}}$ which assume skewed distribution instead of normal distribution [4].

Results

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 [14]. 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 [55].

As compared to electrophysiological membrane capacitance measurements [12, 13], electrochemical amperometric measurement is able to detect exocytosis with single vesicle sensitivity and without interference from endocytosis. Figure 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). The cumulative event number over time (Fig. 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. 1c and 1d, 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. 1e and 1f. Clearly, exocytosis barely recovered 30s after the first intensive stimulation (45.6 ± 4.2%, 8 cells), while it largely restored with 120s’ rest (74.7 ± 10.8%, 8 cells).
PKCε facilitates the recovery of the frequency of exocytotic response

PKCε is a novel PKC isoform expressed abundantly in PC12 cells [51]. It has been shown that it facilitates activity dependent potentiation in bovine chromaffin cells [35] and synaptic potentiation in rat nerve terminal [38]. We herein investigate the roles of PKCε in recovering exocytosis. The dual stimulation protocol with either 30s recovery (Fig. 2a) or 120s recovery (Fig. 2b) was applied to the PC12 cells overexpressed with wt-PKCε, dn-PKCε or vector control.

Although the average exocytosis triggered by the first high K+ stimulation in the cells overexpressed with wt-PKCε 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 vector cells, Fig. 2a and b), such increase is not statistically significant (p > 0.05). On the other hand, overexpression of dn-PKCε led to minor inhibition (34.4 ± 4.0 vesicles, 26 dn cells, p < 0.05). Although overexpression of wt-PKCε 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-PKCε overexpressing cells (Fig. 2a, right). Figure 2c (left) shows the statistics of the recovery percentage for these cells (12 wt cells: 77.2 ± 12.6%; 12 dn cells: 39.4 ± 6.6%, 14 vector cells: 46.2 ± 5.8%). When the vector cells were allowed to rest for 120s, their exocytosis in the second stimulation was largely recovered (Fig. 2b and 2c: 84.9 ± 9.3 %, 12 cells) to an extent similar to the wt-PKCε overexpressing cells (86.3 ± 9.3%, 12 cells). However, recovery of the dn-PKCε overexpressing cells was still suppressed after 120s’ rest (60.8 ± 7.2%, 14 cells). Knockdown of PKCε by siRNA transfection acted similarly as overexpression of dn-PKCε, except that it reduced the efficiency of longer-term (120s) recovery to an extent (44.6 ± 6.5 %, 9 cells) even greater than dn-PKCε inhibition. The negative control of siRNA transfection did not show difference with the vector cells (data not shown). The inhibitory effects of dn-PKCε and siRNA knockdown corroborate the importance of PKCε in exocytotic recovery.

In contrast to PKCε, PKCα did not show facilitating effect on exocytotic recovery (Fig. 2c). The recovery percentages at 30s in wt-PKCα overexpressing cells (46.6 ± 10.6%, 10 cells), the dn-PKCα (47.8 ± 7.8 %, 13 cells), and cells transfected with siRNA against PKCα (54.8 ± 8.8%, 11 cells) are comparable to that in the vector cells (p > 0.05). In addition, as compared to control, there is no significant difference (p > 0.05) in 120s recovery in the dn-PKCα overexpressing cells (n =7: 69.1 ± 14.6%), the wt-PKCα overexpressing cells (n =7: 64.9 ± 14.2%), and the PKCα-siRNA transfected cells (n = 10: 70.0 ± 14.9%). This suggests the regulatory role in exocytotic recovery is not universal to all PKC isoforms.

PKCε mediated fast recovery is likely not due to enhanced Ca2+ signal

There are evidences that PKCs down-regulate Ca2+ current in some cells [23] and up-regulate it in other cell types [9, 15, 27]. We monitored the dynamics of the intracellular Ca2+ concentration to examine whether PKCε facilitates exocytotic recovery by enhancing Ca2+ signal. The intracellular Ca2+ concentration during the dual stimulation protocol was reported by ratiometric fluorescence measurement using a membrane-permeable Ca2+-sensitive dye (Fura2-AM). Figure 3a presents typical Ca2+ responses to the dual high K+ stimulation in vector overexpressing cells. As expected, the elicited Ca2+ signal was transient even the stimulation persisted for long (1 minute) due to inactivation of voltage-activated Ca2+ channels. The exocytotic rundown may be partly attributed to such attenuation of Ca2+ signal [7, 18]. However, Ca2+ current was able to recover largely from inactivation after 30s’ rest (Fig. 3b: 88.7 ± 3.8% in terms of ratiometric measurement of the peak Ca2+ concentration, 7 vector cells), in contrast to the poor recovery of exocytosis within the same time frame (Fig. 2c). Neither overexpression of wt-PKCε nor overexpression of dn-PKCε affected Ca2+ response (both amplitude and time course) to the initial or the second stimulation (30s or 120s later). As demonstrated in Fig. 3b, The Ca2+ recovery in these cells is similar to that in vector cells (30s recovery: 93.1 ± 6.7% from 6 wt cells and 88.1 ± 5.6% from 9 dn cells;
120s recovery: 94.7 ± 6.0% from 5 wt cells and 100.4 ± 3.3% from 6 dn cells). Therefore, it is likely that PKCε regulates fast recovery of exocytosis through Ca^{2+} channel independent pathways. However, we cannot completely rule out the possibility that PKCε may modulate the microdomain Ca^{2+} signal near Ca^{2+} channels which cannot be resolved using our method.

**PKCε mediated recovery is PIP2 dependent**

PIP2 is a membrane phospholipid implicated in multiple aspects of exocytosis [21, 25]. For example, it plays a pivotal role in Ca^{2+} stimulated actin reorganization which is relevant to vesicle trafficking [39]. And PIP2 may be involved in the PKCε pathways via several mechanisms. Firstly, stimulated by membrane depolarization, PIP2 is converted to DAG by phospholipase C (PLC). And DAG subsequently activates PKCε. In addition, it has been shown that PIP2 can interact directly with PKCε [41]. Finally, some substrates of PKCε, such as myristoylated alanine-rich protein kinase C substrate (MARCKS), bind to PIP2 [39]. We examined the roles of PIP2 in PKCε 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 PKCε mediated fast recovery (Fig. 4). The 30s recovery in the neomycin treated wt-PKCε overexpressing cells (n = 7) was only 36.4 ± 8.2%, comparable to that in the untreated vector cells. The neomycin inhibition on the PKCε effect can be partially rescued by PMA (DAG analogue) to 50.1 ± 7.4% recovery (7 wt-PKCε cells treated by both neomycin and PMA). This implies that PKCε mediated fast recovery of exocytosis is achieved partly through PLC-PIP2-DAG-PKCε pathway. On the other hand, both neomycin and PMA gave no obvious influences on dn-PKCε overexpressing cells (Fig. 4).

**PKCε 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 (~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 [54]. Therefore, it is plausible that PKCε mediated fast recovery of exocytosis relies on enhanced rate of vesicle delivery to the plasma membrane.

Individual fluorescently-labeled secretory vesicles near the plasma membrane can be resolved by total internal reflection fluorescence microscopy (TIRFM) which selectively illuminate a thin section (<200 nm) above the interface between the glass coverslip and the adhered cell membrane. We labeled the vesicles in PC12 cells by overexpressing NPY-EGFP and tracked individual vesicles in the subplasmalemmal region at 0.5 s time interval using TIRFM. As revealed by TIRFM imaging, overexpression of both wt- and dn-PKCε 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. 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 cells (Fig. 5a). And in these cells, the delivery rates are similar before (Fig. 5a, left) and after one-minute high K+ stimulation (Fig. 5a, right). Overexpression of wt-PKCε significantly enhanced the rate of vesicle arrival in the absence of stimulation (66.4 ± 2.3 vesicles arrived in two minutes averaged from 5 wt cells vs. 47.6 ± 7.2 vesicles averaged from 5 vector cells, p < 0.01) (Fig. 5a, left). And in these wt cells, the delivery rate was further increased to 94.6 ± 3.1 vesicles right after high K+ stimulation (Fig. 5a, right). The application of neomycin completely blocked the PKCε induced acceleration in vesicle transport both before and after high K+ stimulation (5 neomycin treated wt cells: 48.6 ± 2.3 vesicles before and 43.4 ±
12.0 vesicles after high K\(^+\) stimulation). In comparison to PKC\(\varepsilon\), overexpression of PKC\(\alpha\) also increased the vesicle delivery to 59.8 \(\pm\) 5.4 vesicles (5 cells) before stimulation compared to overexpression of vector, whereas, the vesicle delivery (63.8 \(\pm\) 4.8 vesicles in two minutes) was not further enhanced after stimulation (Fig. 5a). It appears that high K\(^+\) stimulation potentiates the effects of PKC\(\varepsilon\), but not PKC\(\alpha\), in facilitating vesicle trafficking. Overexpression of both dn-PKC\(\varepsilon\) and dn-PKC\(\alpha\) inhibited the rate of vesicle delivery. And stimulation induced potentiation was absent in both dn cells (Fig. 5a). It is also evident that both PKC\(\varepsilon\) and PKC\(\alpha\) do not have obvious influences on the number of vesicles “docked” or “tethered” on the cell membrane (Fig. 5b) despite of their facilitating roles in vesicle trafficking.

**PKC\(\varepsilon\) 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 [46]. 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 [37]. PKC\(\varepsilon\), but not PKC\(\alpha\), was found to be recruited to the vesicle membrane upon Ca\(^{2+}\) stimulation [28]. 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\(\varepsilon\) has been shown to regulate the activity-dependent potentiation of exocytosis via actin rearrangement [35].

Cortical actin network was fluorescently stained and examined by confocal imaging in the wt- or dn-PKC\(\varepsilon\) overexpressing PC12 cells without or right after one-minute high-K\(^+\) stimulation. It is evident from Fig. 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 15.1 \(\pm\) 2.1\% averaged from 457 control cells to 55.6 \(\pm\) 4.3\% averaged from 412 control cells) after high K\(^+\) stimulation (Fig. 6b) because Ca\(^{2+}\) influx that accompanies membrane depolarization induces actin disassembly [33, 47]. The observation that overexpression of dn-PKC\(\varepsilon\) greatly inhibited the post-depolarization disassembly of cortical actin (Fig. 6b, 29.9 \(\pm\) 2.3\% fragmentation, 141 cells) is consistent with the previous report in which membrane depolarization is induced by nicotine in mouse chromaffin cells [35]. On the other hand, overexpression of wt-PKC\(\varepsilon\) enhanced actin disassembly after high K\(^+\) stimulation (79.7 \(\pm\) 3.2\% fragmentation, 124 cells). Such enhancement was blocked by neomycin (38.5 \(\pm\) 7.1\% fragmentation, 114 cells), suggesting the critical involvement of PIP2. In comparison, the increase of actin fragmentation after high K\(^+\) stimulation in the wt-PKC\(\alpha\) overexpressing cells was less prominent (37.2 \(\pm\) 0.72\% from 94 cells without high K\(^+\) stimulation vs. 54.7 \(\pm\) 2.7\% from 94 cells after high K\(^+\) stimulation). It is likely that PKC\(\varepsilon\) regulation on actin dynamics underlies the PKC\(\varepsilon\) mediated fast vesicle delivery and fast recovery of exocytosis.

**PKC\(\varepsilon\) facilitates the recovery of the kinetics of quantal vesicle release**

It is notable from Fig. 1a and 1c 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 quantal vesicle release. 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 \(\pm\) 0.56 pA to 12.2 \(\pm\) 0.92 pA (14 cells, Fig. 7a). Longer recovery period (120s) did not help to restore the amperometric amplitude (Fig. 7a: 11.18 \(\pm\) 0.57 pA, 12 cells) despite that the extent of exocytosis was regained to \(\sim\)84.9\% of the original competence (Fig. 2c).
The time it takes for the amperometric signal to rise from 35% to 90% of the peak amplitude ($t_{\text{rise}}$) reflects how fast the initial fusion pore formed between the vesicle membrane and the plasma membrane expands to discharge the vesicular cargo [3, 17]. The average $t_{\text{rise}}$ 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. 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 vesicular release can be characterized by the half-width time of the amperometric spike ($t_{1/2}$) [3, 17]. As demonstrated in Fig. 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 release 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. 7d). All these observations suggest that rundown in the kinetics (or efficiency) of quantal vesicular release kinetics occurs.

Investigation on the recovery of release kinetics was similarly carried out on the cells overexpressed with wt-PKCε or dn-PKCε. Overexpression of wt-PKCε (12 cells) enabled almost full recovery in the fusion kinetics in 30s ($I_{\text{max}} = 17.45 ± 1.11$ pA, $t_{\text{rise}} = 1.40 ± 0.06$ ms and $t_{1/2} = 2.63 ± 0.09$ ms). It is also evident from the ratios of $I_{\text{max}}$, $t_{\text{rise}}$ and $t_{1/2}$ between the average from the second response and that from the first response (Fig. 7e), i.e., they are closer to 1 as compared to those from the vector control cells. As anticipated, overexpression of dn-PKCε showed inhibitory effects in restoring the kinetics of exocytotic events. In this case (12 cells), the ratios of $I_{\text{max}}$, $t_{\text{rise}}$ and $t_{1/2}$ are 51.8 ± 5.2%, 155.4 ± 13.7% and 169.3 ± 9.0%, respectively. siRNA knockdown is similarly inhibitory (Fig. 7e). All these observations suggest the importance of PKCε in recovery of quantal vesicular release.

Intriguingly, the PKCε facilitation on recovery of quantal release appears to be short-term because overexpression of wt-PKCε did not make significant difference after 120s’ rest as compared to the vector control (Fig. 7f). The average amperometric spikes responding to the first or the second stimulation (30s or 120s later) from the wt cells and the dn cells are displayed in pair in Fig. 7g. The difference after 30s recovery is pronounced (Fig. 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-PKCε overexpressing cells was much better while that in the dn-PKCε overexpressing cells was significantly worse. These observations clearly demonstrate the facilitating role of PKCε in fast recovery of quantal release. In contrast to PKCε, PKCα plays a significant role in supporting the amperometric response to the first stimulation. As shown in Fig. 7g, in response to the first stimulation, overexpression of wt-PKCα (17 cells) significantly enhanced the $I_{\text{max}}$ (to 121.4 ± 6.0 % of vector control, $p < 0.01$) whereas overexpression of dn-PKCα (20 cells) and transfection of siRNA against PKCα (21 cells) significantly inhibited the $I_{\text{max}}$ (to 61.1 ± 4.1 % and 42.4 ± 4.1 % of vector control, respectively; $p < 0.001$). On the other hand, overexpression of wt-PKCα did not give better recovery compared to the control, overexpression of dn-PKCα, or transfection of PKCα-siRNA. These observations demonstrate the distinct roles of PKCε and PKCα in regulating the process of quantal vesicular release.

**Discussion**

Multiple PKC isoforms have been identified in neuroendocrine PC12 cells. Among them, PKCε is expressed most abundantly and PKCα is the most active [51]. Based on overexpression of wt-PKCε or its dominant negative, we herein provide evidences that PKCε, but not PKCα, facilities the recovery of exocytotic rate and kinetics of quantal vesicular release 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 may suggest a PKC-dependent mechanism relevant to the short-term plasticity of exocytosis in both neurons and neuroendocrine cells.

We show that long-lasting stimulation to PC12 cells causes rundown not only in the frequency of exocytotic events but also in the kinetics (specifically, smaller amperometric amplitude, slower fusion pore expansion and longer release 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 [11, 30] or/and inactivation of Ca$^{2+}$ current [7, 18]. As quantal release kinetics has been found to be Ca$^{2+}$ concentration dependent [48], slowdown in release kinetics may be due in part to Ca$^{2+}$ inactivation as well. But, as demonstrated in Fig. 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. 2c and 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 [49]. 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 quantal kinetics is not synchronic. Specifically, in control cells, the extent of exocytosis recovers to ~80% in 120s while kinetics of quantal exocytotic events remains slow. It may imply the mechanistic difference in these two recovery processes. Further experiments are however needed to address this question.

Wt-PKCε overexpressing cells only needs ~30s to restore to ~80% of the normal exocytotic competence after an exhausting stimulation, while 30s recovery of the dn-PKCε overexpressing cells is only ~39%, worse than the vector control (~46%). PIP2-sequestering neomycin can completely eliminate PKCε mediated fast recovery, suggesting the crucial involvement of PIP2. Based on these observations, the following scenario may be speculated. Membrane depolarization and/or Ca$^{2+}$ influx activates PLC [44] which hydrolyzes PIP2 into DAG. In turn, DAG recruits and activates PKCε. PKCε activation then increases the release probability of the vesicle through phosphorylation of the serectroy proteins relevant to vesicle docking, priming and fusion, such as SNAP-25 [32, 40], synaptotagmin [20, 22], and Munc18 [5, 19, 34]. At the same time, Ca$^{2+}$ 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 PKCε [1].

Since fusion proteins such as SNAP-25 are PKCε targets, it should not be unexpected that PKCε facilitates the recovery of quantal release kinetics. However, such PKCε facilitation appears to be short-term. As demonstrated in Fig. 7, in wt-PKCε overexpressing cells, release kinetics recovered mostly in 30s whereas PKCε 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 [55]. It has been shown that post-tetanic potentiation critically relies on enhanced Ca$^{2+}$ sensitivity of vesicle fusion mediated by PKC [8]. In addition, PKCε 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 [6, 16]. We tested the effect of neomycin on PKCε mediated recovery of the kinetics of quantal exocytotic events (data not shown). Although overexpression of PKCε showed no facilitation under neomycin treatment, unequivocal
conclusion cannot be drawn because neomycin treatment itself significantly impairs the release kinetics (smaller amperometric amplitude, longer $t_{rise}$ and $t_{1/2}$).

PKCε and PKCα differentially regulate exocytosis in PC12 cells. Compared to the vector control, overexpression of both wt-PKCe and wt-PKCa did not significantly augment the extent of exocytosis responding to the first (single) stimulation, whereas overexpression of dn-PKCa gave more severe inhibition compared to dn-PKCe (21 dn-PKCa cells: $66.3 \pm 8.0\%$ of the control response, $p < 0.01$ vs. 26 dn-PKCe cells: $79.8 \pm 9.2\%$, $p < 0.05$ ). Overexpression of both wt-PKCe and wt-PKCa increased the amperometric amplitude. But the enhancement by PKCa was more pronounced (17 wt-PKCa cells: $121.4 \pm 6.0\%$ of the control response, $p < 0.01$ vs. 24 wt-PKCe cells: $112.1 \pm 4.2\%$, $p < 0.05$). These observations suggest the facilitating roles of PKCa on the exocytosis triggered by initial stimulation are more prominent compared to that of PKCe. However, PKCa facilitations disappeared in the exocytotic response to the second stimulation (i.e., not significantly different from control). On the other hand, wt-PKCe gave significant enhancement on exocytotic response to the second stimulation (30s after the first). In other words, it is PKCe, but not PKCa, that facilitates the fast recovery of the extent of exocytosis and the kinetics of quantal release. Activation of PKCa is Ca$^{2+}$ dependent. The actions of PKCa seem to synchronize with the initial stimulation (and accompanying Ca$^{2+}$ signal) while the actions of PKCe, whose activation does not directly require Ca$^{2+}$, seem to be delayed. And effects of both PKCa and PKCe are transient. 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.

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Figure Legends

Fig. 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 (dark curve) and the second (gray curve) 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.

Fig. 2 PKCe 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. 1c). The average responses to the first stimulation from 14 vector overexpressing cells (vector, gray), 12 wt-PKCe overexpressing cells (wt, dark) and 12 dn-PKCe overexpressing cells (dn, 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. 1d), averaged from 12 vector cells, 12 wt cells, and 14 dn 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 ± SEM, with the recovery period of 30s (from the cell groups used in a, 11 cells transfected with siRNA against PKCe, 10 wt-PKCa and 13 dn-PKCa overexpressing cells, 11 cells transfected with siRNA against PKCa) or with recovery period of 120s (from the cell groups used in b, 9 anti-PKCe siRNA transfected cells, 7 wt-PKCa and 7 dn-PKCa overexpressing cells, 10 anti-PKCa siRNA transfected cells). Unpaired Student’s t-test with reference to the vector control: *p<0.05, ** p<0.01.

Fig. 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 (Fura2) 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 ± SEM. The data on 30s recovery (left) is based on the recordings from 7 vector cells, 6 wt cells, and 9 dn cells. The data on 120s recovery (right) is based on the recordings from 6 vector cells, 5 wt cells, and 6 dn cells.

Fig. 4 Neomycin impairs PKCe mediated fast recovery of exocytosis (30s recovery). The statistics of the recovery percentage is shown as mean ± SEM. The data of untreated wt (column 1) or dn cells (column 4) is the same as in Fig. 2c. Recovery in wt cells (n = 7) is significantly inhibited after pre-treatment of neomycin (NEO) (column 2). Application of PMA to the same neomycin-pretreated wt 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 cells (n = 8). The dash line represents the mean recovery percentage from untreated vector cells as shown in Fig. 2c. Unpaired Student’s t-test: *p<0.05, **p<0.01. Paired Student’s t-test: +p<0.05.

Fig. 5 PKCe mediates vesicle delivery from the inner cytosol to the subplasmalemmal region. The data is averaged from 5 vector cells (open circle), 8 wt-PKCe cells (open square), 6 dn-PKCe cells (filled square), 5 NEO-pretreated wt-PKCe cells (cross), 5 wt-PKCa cells (open triangle), 5 dn-PKCa 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. 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.

Fig. 6 PKCε 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 sale bar = 5 μm. b The percentage of cells that have fragmented cortical actin network (as shown on right in a) under different conditions. Fragmentation is defined when the broken portion of the cortical ring is >20%. Each data represented by mean ± SEM was from > 110 cells in 5 independent experiments. Unpaired Student’s t-test: **p<0.01, ***p<0.001 (reference is indicated by brackets); #p<0.01 (reference to control after high K⁺, column 2).

Fig. 7 PKCε facilitates the recovery of fusion kinetics. Analyses are based on the same data set presented in Fig. 2. a-d The statistics of amplitude (I_m), 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 ± SEM. I_m 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_m (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_m, t_rise and t_1/2 (mean value obtained from the second stimulation divided by that from the first stimulation) are shown as mean ± 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) PKCε (top row) or PKCα (bottom row) cells, in response to the first stimulation (left: 1121 spikes from 26 vector cells; left-top: 1205 spikes from 24 wt-PKCε cells, 894 spikes from 26 dn-PKCε cells; left-bottom: 460 spikes from 17 wt-PKCα cells, 365 spikes from 20 dn-PKCα 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-PKCε cells, 152 spikes from 12 dn-PKCε cells; middle-bottom: 133 spikes from 10 wt-PKCα cells, 104 spikes from 13 dn-PKCα cells) or after 120s of recovery (right: 389 spikes from 12 vector cells; right-top: 489 spikes from 12 wt-PKCε cells, 254 spikes from 14 dn-PKCε cells; right-bottom: 68 spikes from 7 wt-PKCα cells, 100 spikes from 7 dn-PKCα cells).
Fig. 1
Fig. 3

(a) Time course of F340/F380 ratio for different treatments.

(b) Recovery of F340/F380 ratio over time with different treatments.

Fig. 4

Graph showing % of recovery with different treatments.

Legend:
- vector
- wt-PKCε
- dn-PKCε

Treatments:
- NEO
- PMA

Symbols:
- * significance
- ** high significance
- + slight significance
Fig. 5

(a) Without high K^+:
- Vector
- wt-PKCα
- dn-PKCα
- wt-PKCα-NEO
- wt-PKCα
- dn-PKCα

(b) Without high K^+:
- Vector
- wt-PKCα
- dn-PKCα

Graphs show the number of arrived vesicles and subplasmalemmal vesicles over time before and after high K^+.
Fig. 6

(a) Images showing...

(b) Bar graph representing...

- % of F-actin fragmentation

- Control, wt-ε, dn-ε groups

- Before high K⁺, after high K⁺, NEO and after high K⁺
Fig. 7

(a) $l_{max}$ (pA) for different treatments and time points (1st, 30s, 120s).

(b) $t_{rise}$ (ms) for different treatments and time points (1st, 30s, 120s).

(c) $t_{1/2}$ for different treatments and time points (1st, 30s, 120s).

(d) Q (TC) for different treatments.

(e) Ratio of $l_{max}$ for different treatments (vector, wt-ε, dn-ε, siRNA-ε) at Δτ = 30s.

(f) Ratio of $l_{max}$ for different treatments (vector, wt-ε, dn-ε, siRNA-ε) at Δτ = 120s.

(g) 1st and 2nd Stimulated responses for different treatments (wt-PKCε, dn-PKCε, vector) at Δτ = 30s, 120s.