Cellular/Molecular

Involvement of Protein Kinase C-ε in Activity-Dependent Potentiation of Large Dense-Core Vesicle Exocytosis in Chromaffin Cells

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Neurotransmitter release is modulated in an activity-dependent manner. We showed previously that repetitive stimulation of nicotinic acetylcholine receptor (nAChR) induced activity-dependent potentiation (ADP) of large dense-core vesicle (LDCV) exocytosis in chromaffin cells. Here we report that protein kinase C (PKC)-ε is critically involved in ADP. Stimulation of nAChR induced activation of PKC-ε, and inhibition of PKC-ε by expression of the dominant-negative mutant of PKC-ε (DN-PKC-ε) or short interfering (siRNA) against PKC-ε abolished ADP via decreasing the frequency and quantal size of fused vesicles without affecting basal exocytosis, suggesting that PKC-ε is specifically involved in ADP. Electron microscopy revealed that inhibition of PKC-ε disrupts activity-induced vesicle translocation required for ADP. We also suggest the involvement of myristoylated alanine-rich C kinase substrate (MARCKS), which is known as a downstream target of PKC-ε, in ADP of LDCV exocytosis. The level of phospho-MARCKS correlated with the time course of ADP and was reduced by transfection with DN-PKC-ε. Actin filament disassembly induced by MARCKS phosphorylation was also significantly blocked by transfection of DN-PKC-ε. Furthermore, knockdown of MARCKS by siRNA resulted in inhibition of ADP and reduction of the number of fused vesicles. Together, we provide evidence that ADP of LDCV exocytosis is regulated by PKC-ε and its downstream target MARCKS via modulating vesicle translocation.

Key words: **LDCV; PKC-ε; MARCKS; activity-dependent potentiation; amperometry; neurotransmitter**

Introduction

Large dense-core vesicles (LDCVs), which contain neurotransmitters of amines and peptides, play a critical role in the regulation of the autonomic nervous system. Exocytosis of neurotransmitters is a highly regulated process that requires translocation of vesicles to the plasma membrane, followed by vesicle fusion triggered by elevation of intracellular calcium concentration. Increases in transmitter release induced by successive and repetitive stimulation represent activity-dependent potentiation (ADP) resulting from increased vesicle translocation. It has been reported that release of LDCVs from neuromuscular junctions and neuroendocrine cells shows ADP (Engisch et al., 1997; Smith, 1999; Shakiryanova et al., 2005), whereas activity-dependent depression of exocytosis is usually dominant in excitable cells that likely reflects a depletion of a readily releasable pool (RRP) of vesicles (Zucker and Regehr, 2002). We have shown previously that repetitive stimulation of nicotinic acetylcholine receptor (nAChR) gives rise to ADP of exocytosis, which had calcium dependence and resulted from increased vesicle translocation (Park et al., 2006). However, the molecular mechanisms by which ADP of LDCV release can be regulated are essentially unknown.

Protein kinase C (PKC) has been implicated in enhancement of neurotransmitter release by increasing the size of the RRP (Gillis et al., 1996; Majewski and Iannazzo, 1998; Smith, 1999). PKC isoforms, referred to conventional, novel, and atypical types, are activated by diacylglycerol (DAG), phosphatidylserine, and/or calcium ions (Battaini, 2001). PKC-ε, a novel type of PKC that is a predominantly expressed isoform in neuronal cells (Tanaka and Nishizuka, 1994), is selectively anchored to actin filament (F-actin), suggesting that PKC-ε might be involved in the replenishment of small synaptic vesicles via controlling F-actin dynamics (Prekeris et al., 1996). However, the role of PKC in translocation of LDCVs, which was shown to be required for ADP, has not been elucidated.

Myristoylated alanine-rich C kinase substrate (MARCKS) is a well known substrate of PKC and plays a key role in cytoskeleton dynamics, cell motility, and exocytosis (Arbuzova et al., 2002). MARCKS is recruited to the plasma membrane via its N-terminal myristoylation and electrostatic interactions with acidic membrane phospholipids. Phosphorylation of MARCKS by PKC disrupts the electrostatic interactions between MARCKS and the plasma membrane, thereby inducing translocation of MARCKS to the cytosolic fraction (Arbuzova et al., 2002). In addition,

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phosphorylation of MARCKS also results in F-actin disassembly, which is associated with vesicle recruitment to the plasma membrane (Rose et al., 2001).

In the present study, we investigated the involvement of PKC-ε as well as its downstream molecule MARCKS in ADP of LDCV release. Here we provide evidence that activation of PKC-ε is responsible for ADP of LDCV release via increasing vesicle translocation, the frequency of vesicle fusion, and the quantal size of vesicles. We also suggest that MARCKS, which is phosphorylated by PKC-ε, is critically involved in ADP of LDCV release through regulating F-actin disassembly and vesicle translocation.

Materials and Methods

Materials. LysoTracker Green, LysoTracker Red, and rhodamine phalloidin were from Invitrogen (Carlsbad, CA). 1,1-Dimethyl-4-phenylpiperazinium iodide (DMPP), sucrose, and GF109203X (3-[1-(3-dimethylaminopropyl)indol-3-yl]-4-(1*H*-indol-3-yl)pyrrole-2,5-dione) were purchased from Sigma (St. Louis, MO). Ro-31-8220 (2-[1-(3-(amidinothio)propyl)-1*H*-indol-3-yl]-3-(1-methylindol-3-yl)maleimide) and Gö6976 (12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo[2,3-a]pyrrolo[3,4-c]carbazole) were from Calbiochem (San Diego, CA). [γ-^{32}P]ATP was obtained from NEN (Boston, MA). Anti-PKC-ε and anti-synaptotagmin monoclonal antibody were from Transduction Laboratories (Lexington, KY). Anti-tyrosine hydroxylase (TH) and anti-phospho-MARCKS polyclonal antibody were from Cell Signaling Technology (Beverly, MA). The enhanced chemiluminescence (Supex) kit was from Neuronex (Pohang, Korea).

Preparation of bovine chromaffin cells. Chromaffin cells were isolated from bovine adrenal gland medulla by two-step collagenase digestion as described previously (Park et al., 2006).

Amperometric measurement. We monitored the release of LDCVs, which contain catecholamine in real time (Chow et al., 1992). For analysis of amperometric spikes, e.g., frequency, amplitude (I_{max}), half-width ($t_{1/2}$), charge (Q), rising slope (amperes per second), falling slope (amperes per second), and prespike foot of amperometric signals, Igor software (WaveMetrics, Lake Oswego, OR) was used with a macro program (Mosharov and Sulzer, 2005). We discarded amperometric spikes that were overlapped or under 2.5 pA amplitude. Recordings of chromaffin cells, which were grown on poly-D-lysine-coated glass coverslips, were performed at room temperature as described previously (Park et al., 2006).

Recombinant adenoviruses. Recombinant adenovirus expressing internal ribosomal entry site–enhance green fluorescent protein (IRES–EGFP) and the dominant-negative mutant of PKC-ε (DN-PKC-ε) conjugated with IRES–EGFP were developed by using pShuttle–IRES–humanized *Rotylenchulus reniformis* GFP and pAdEasy-1 vector (Stratagene, La Jolla, CA). DN-PKC-ε was a gift from Dr. Y. S. Lee (Korea Cancer Center Hospital, Seoul, Korea). BJ5183 and HEK293T cells were used for homologous recombination and preparation of adenovirus, respectively, as described previously (He et al., 1998). Adenovirus containing DN-PKC-ζ conjugated with IRES–EGFP was provided by Dr. J. S. Chun (Gwangju Institute of Science and Technology, Gwangju, Korea). Chromaffin cells were transfected with adenovirus (10 multiplicity of infection) for 24–36 h, as described in detail previously (Li et al., 2002).

RNA interference. Short interfering RNA (siRNA) duplexes for knockdown of PKC-ε (5′-UAC GAA GUG UGC UGG AUU AUU-3′ and 5′-AGA CUC AAC UAC UGU UAU UUU-3′), PKC-δ (5′-UAC AGU UUC UGC ACA GAA AUU-3′ and 5′-ACA UCG AGA UUG CCG ACU UUU-3′), and MARCKS (5′-CCA GCA AGG CUG AAG AGA AUU-3′ and 5′-UGA GCG GCU UCU CCU UCA AUU-3′) expression were purchased from Dharmacon (Chicago, IL). Scrambled nontargeting siRNA was used as a negative control. Transfection with siRNA pools was performed by Lipofectamine reagent (Invitrogen) with enhancing peptide (JBI, Daegu, Korea). Downregulation of MARCKS was confirmed by quantitative reverse transcription (RT)-PCR. For quantitative RT-PCR, cellular RNA was extracted from the mock and MARCKS siRNA-expressing cells, and 2 μg of total RNA was reverse transcribed into cDNA by Moloney murine leukemia virus reverse transcriptase (Roche Molecular Biochemicals, Mannheim, Germany). Primers used in PCR were as follows: PKC-ε, forward, 5′-ATTGCAAGCTGGCTGACTTT-3′ and reverse, 5′-GTCGTCATGGAGGATGGACT-3′; PKC-δ, forward, 5′-GCACAGAAAGGGCATCATTT-3′ and reverse, 5′-TCAGGGGC-GATATAGTCAGG-3′; MARCKS, forward, 5′-GGCCGGCTCATCTT-TCTC-3′ and reverse, 5′-GTCGCCTTCTAAAGCGAATG-3′; and TH, forward, 5′-GATGCCAAGGACAAGCTCA-3′ and reverse, 5′-CTGA-TGGCGTTCAGAGCAT-3′.

Cell fractionation and immunoblotting. Cells stimulated as indicated were washed with chilled PBS. To separate the cell lysates into the cytosolic and membrane fraction, cells were suspended in buffer A (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 mM EGTA, 10 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM dithiothreitol, 2 mM ascorbic acid, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Cell lysates were sonicated and centrifuged at 100,000 × g for 1 h, and the supernatant was saved as the cytosolic fraction. The pellet was then resuspended in buffer A and saved as the membrane fraction. Proteins were then separated by electrophoresis containing 0.1% SDS and transferred to a nitrocellulose membrane, which was blocked with 5% nonfat dry milk in TTBS solution (20 mM Tris-HCl, pH 7.4, 140 mM NaCl, and 0.05% Tween 20). Phospho-MARCKS, TH, and synaptotagmin were detected with specific antibodies and horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology). Bands were visualized with the ECL detection system (Neuronex).

Kinase assay. Activity of PKC-ε was determined by the immune complex kinase assay as described previously (Lee et al., 2003). Cell lysates were prepared in lysis buffer and incubated with a monoclonal antibody against PKC-ε for 2 h. Immune complexes were collected on protein G-Sepharose beads (Amersham Biosciences, Uppsala, Sweden). The beads were then resuspended in kinase reaction buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerol phosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, [γ-^{32}P]ATP, and 1 μg of histone1). Substrate phosphorylation was detected by autoradiography.

Electron microscopy. Electron microscopy was performed as described in detail previously (Park et al., 2006).

F-actin disassembly. Staining of F-actin was performed in chromaffin cells as described previously (Rose et al., 2001; Park et al., 2006). After washout with PBS, cells were stained for F-actin with 0.5 U/ml rhodamine–phalloidin for 20 min at room temperature. Each cell examined was classified as having either a "continuous" (indicating F-actin) or "discontinuous" (indicating F-actin disassembly) cortical rhodamine fluorescent ring.

Statistical analysis. All quantitative data are presented as means ± SEM. Comparison between two groups were analyzed by using Student's t test, and values of $p < 0.05$ were considered to be significant. To compare results between more than three groups, we used a one-way ANOVA with significance level of $p < 0.05$ (Origin 6.0 program; Microcal Software, Northampton, MA).

Results

Repetitive activation of nAChR induces ADP of LDCV exocytosis and PKC-ε translocation to the plasma membrane

Amperometry has been used to monitor the release of catecholamine stored in LDCVs (Park et al., 2006). Repetitive stimulation of chromaffin cells with DMPP, a selective agonist of nAChRs, induced ADP of LDCV release (Fig. 1*A*,*B*), as we reported previously (Park et al., 2006). Our recent study suggested that the effect of ADP is attributed to the increased translocation of vesicles to the plasma membrane. Because PKC has been implicated in vesicle translocation (Shoji-Kasai et al., 2002; Kumakura et al., 2004), we examined whether PKC is involved in ADP of LDCV release. Inhibitors of pan-PKC isoforms (GF109203X and Ro-31-8220) blocked ADP, in contrast to an inhibitor of classical type PKC isoforms (Gö6976), suggesting involvement of a nonclassical type of PKC in ADP. Bovine chromaffin cells express several

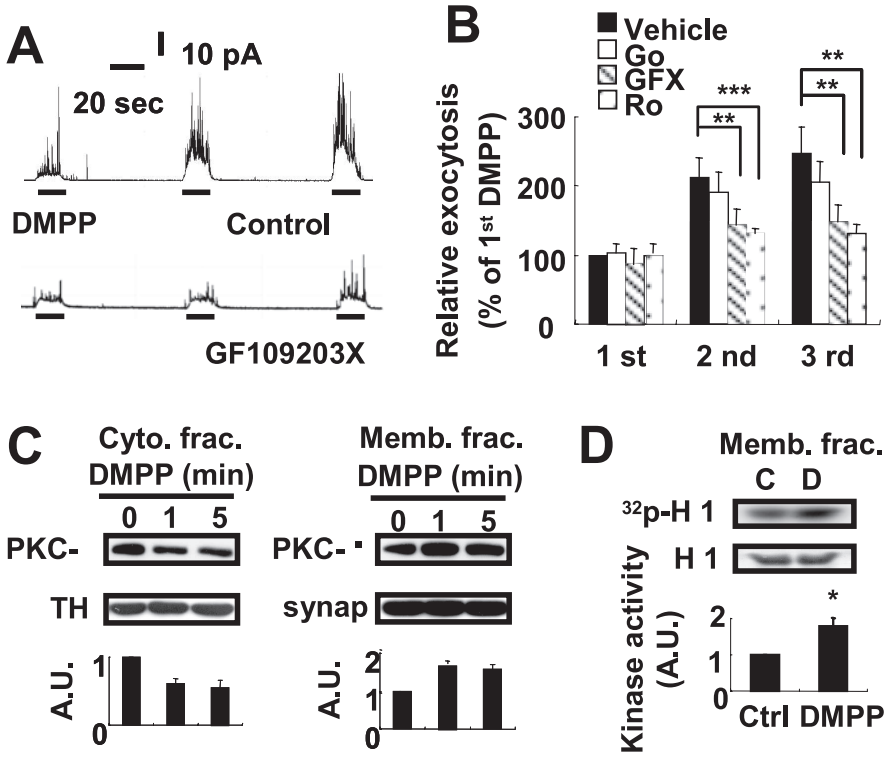

Figure 1. Activation of nAChR induces ADP and translocation of PKC-ε. ***A***, Inhibition of PKC attenuates ADP of exocytosis. Chromaffin cells were repetitively stimulated with 10 μM DMPP under pretreatment of vehicle (top; $n = 9$) or GF109203X (bottom; $n = 8$) for 3 min. Shown is a representative trace recorded by amperometry. Duration of each stimulus was 20 s, and the interval time between stimuli was 2 min. ***B***, Involvement of nonclassical PKC isoforms in ADP of exocytosis. DMPP was repetitively applied, under preincubation of pan-PKC isoforms inhibitors, 1 μM GF109203X (GFX; $n = 8$) and 1 μM Ro-31-8220 (Ro; $n = 6$), or a classical type PKC inhibitor, 1 μM Gö6976 (Go; $n = 6$). Amperometric current generated by repetitive stimulation was individually integrated. Relative exocytosis is presented as a percentage value of the first stimulation-induced total catecholamine release obtained from vehicle-treated cells. Data are presented as mean ± SEM, and significance is taken using one-way ANOVA. ***C***, nAChR stimulation induces translocation of PKC-ε into the plasma membrane fraction. Cells were treated with DMPP for the indicated times, followed by washout with Ringer's solution for 1 min, and then fractionated into the cytosolic fraction (Cyto. frac.) and the membrane fraction (Memb. frac.). Samples were immunoblotted by anti-PKC-ε antibody. Immunoblotting with the TH and synaptotagmin (synap) antibody was performed for normalization of the cytosolic and membrane fraction, respectively. ***D***, PKC-ε translocation indicates activation of PKC-ε in the membrane fraction. Stimulation with DMPP induces PKC-ε translocation and thereby activates PKC-ε obtained from the membrane fraction. Cells were exposed to 10 μM DMPP for 1 min and then washed out with Ringer's solution for 1 min. After fractionation, activity of PKC-ε obtained from the membrane fraction was determined by a kinase assay, as described in Materials and Methods. Kinase activity and immunoblot were quantified by densitometry and normalized as an arbitrary unit (A.U.). Data represented in ***B–D*** are means ± SEM. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

types of PKC isoforms: classical (α and β), novel (ε and δ), and atypical (ι) type (data not shown). Among the nonclassical types of PKC isoforms, PKC-ε has been implicated in the regulation of exocytosis in synapses (Prekeris et al., 1996). In addition, the protein or mRNA expression level of PKC-ε is very high compared with that of PKC-δ (Fig. 2*E*). Therefore, we examined the involvement of PKC-ε in ADP of LDCV release. As shown in Figure 1*C*, stimulation of nAChRs induced translocation of PKC-ε to the membrane fraction. Moreover, kinase assay revealed that the increased translocation of PKC-ε correlates with its activation (Fig. 1*D*). We could not detect any kinase activity of PKC-ε in the cytosolic fraction (data not shown), although a substantial amount of PKC-ε was detected (Fig. 1*C*). Collectively, these results suggest that stimulation of nAChR induces activation of PKC-ε in the plasma membrane.

PKC-ε mediates ADP of LDCV exocytosis

To investigate whether PKC-ε regulates ADP, we transfected chromaffin cells with adenovirus expressing DN-PKC-ε. The extent of ADP was substantially reduced in cells expressing DN-PKC-ε compared with cells expressing EGFP alone (Fig. 2*B*). Importantly, expression of DN-PKC-ε did not have any effect on the LDCV release induced by the first stimulation, suggesting that PKC-ε was specifically involved in the potentiation process (Fig. 2*B*). We showed previously that both the area of amperometric current (representing the total amount of released catecholamine) and the number of spikes (representing fused vesicles) increased during repetitive stimulation (Park et al., 2006). Expression of DN-PKC-ε inhibited the increase in spike number (Fig. 2*C*), as well as the total amount of catecholamine (Fig. 2*B*). Hypertonic sucrose solution evokes exocytosis in a calcium-independent manner and cannot cause ADP of exocytosis (Park et al., 2006). As a control, we observed that DN-PKC-ε did not affect exocytosis induced by repetitive stimulation with hypertonic sucrose solution (Fig. 2*D*), suggesting that DN-PKC-ε did not disrupt the basal exocytosis. In addition, inhibition of PKC-ε by either treatment with GF109203X or expression of DN-PKC-ε did not change the DMPP-induced calcium influx (data not shown).

To further confirm the involvement of PKC-ε in ADP of exocytosis, we used a RNA interference approach. siRNA against PKC-δ, another novel type PKC, was tested as a negative control (Fig. 2*E*). Knockdown of PKC-ε by transfection with siRNA inhibited ADP compared with the level in scrambled siRNA or PKC-δ siRNA-transfected cells (Fig. 2*F*). Altogether, we suggest that PKC-ε mediates ADP, without affecting the basal exocytosis and calcium influx.

PKC-ε regulates the spike frequency and quantal size

Next, we analyzed the fusion pore kinetics of amperometric spikes modulated by repetitive stimulation. The second stimulation of DMPP increased the spike frequency compared with the first stimulation (Fig. 3*B*). Especially, the ratio of increase in spikes number was greater in large spikes (>20 pA of peak amplitudes) (Fig. 3*B*,*C*). Also, repetitive stimulation modulates fusion pore kinetics, as demonstrated by averaged amperometric spikes (Table 1). Repetitive stimulation increased the I_{max}, rise slope, and quantal size but reduced the half-width of spikes. In addition, the foot I_{max} and foot charge were increased, but repetitive stimulation had no significant effect on the foot frequency (Table 1). Inhibition of PKC-ε by either DN-PKC-ε or siRNA against PKC-ε reduced the increase of both the frequency and number of large spikes (Fig. 3*E*,*F*). In addition, repetitive stimulation-induced changes in fusion pore kinetics, including the quantal size, were reversed by expression of DN-PKC-ε (data not shown). Together, we suggest that PKC-ε mediates ADP of

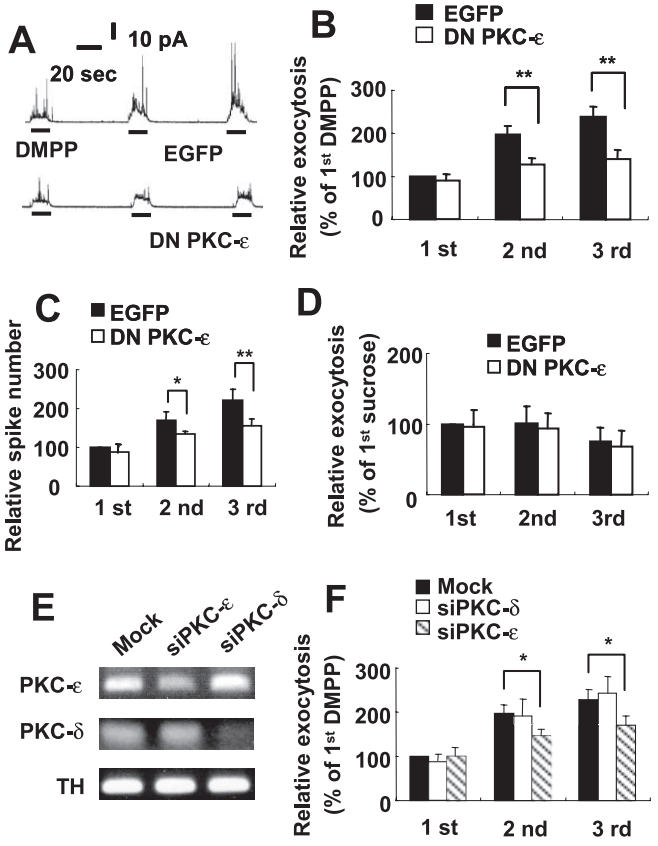

Figure 2. PKC-ε is responsible for ADP. ***A***, Expression of dominant-negative PKC-ε inhibits ADP of exocytosis. Typical traces of amperometry are shown from cells infected by adenovirus containing either IRES–EGFP alone (top) or dominant-negative PKC-ε conjugated with IRES–EGFP (bottom). ***B***, Relative exocytosis of transfected cells with EGFP alone ($n = 9$) or dominant-negative PKC-ε ($n = 10$) was presented as a percentage of the first DMPP-induced catecholamine release obtained from EGFP alone-transfected cells. ***C***, Dominant-negative PKC-ε reduces the spike number of amperometric current, which is potentiated by repetitive stimulation. Relative spike number of amperometric current obtained from the result in ***B*** is presented as a percentage of the first stimulation-induced spike number from EGFP alone-transfected cells. ***D***, Dominant-negative PKC-ε does not inhibit exocytosis evoked by repetitive stimulation with hypertonic sucrose solution. Hypertonic sucrose solution at 500 mM was repetitively applied instead of DMPP to EGFP ($n = 5$) or dominant-negative PKC-ε ($n = 5$) transfected cells. ***E***, Knockdown of PKC-ε inhibits ADP of exocytosis. Expression of PKC-ε and PKC-δ mRNA in the mock- and siRNA-transfected cells was analyzed by quantitative RT-PCR. TH expression is presented as a control for equal amount of cDNA. ***F***, Relative exocytosis of transfected cells with the mock ($n = 7$), PKC-ε siRNA ($n = 8$), and PKC-δ siRNA ($n = 5$) was presented as a percentage of the first DMPP-induced catecholamine release obtained from the mock-transfected cells. We identified transfected cells as EGFP fluorescent signaling. Data presented in ***B–D*** and ***F*** are means ± SEM values. $^*p < 0.05$; $^{**}p < 0.01$. Significance of data presented in ***F*** is taken using one-way ANOVA.

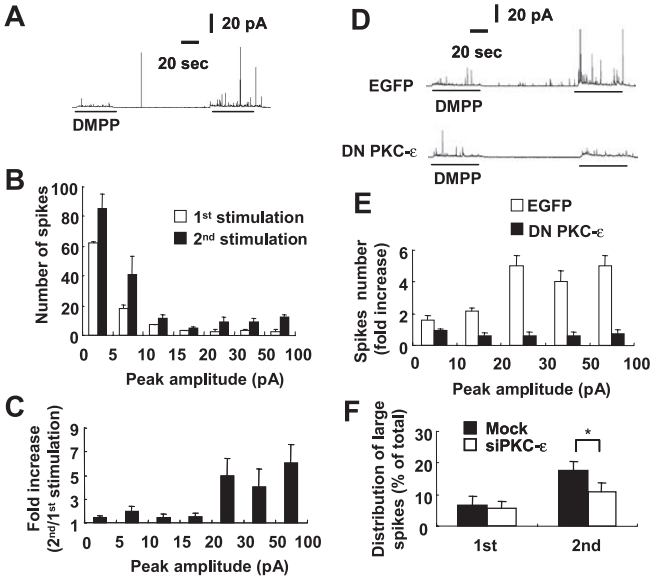

Figure 3. Expression of DN-PKC-ε inhibits the increases in the spike frequency and amplitude. ***A***, Chromaffin cells were repetitively stimulated with 10 μM DMPP for 1 min with 2 min intervals ($n = 5$). Shown is a representative trace recorded by amperometry. ***B***, The distribution of quantal size of amperometric spikes with different peak amplitude induced by the first and second DMPP stimulation. The second DMPP stimulation (black) increases the number of amperometric spikes compared with the first stimulation (white). ***C***, Spikes that have large peak amplitude (>20 pA) significantly increases during the second stimulation. Fold increase is presented as a ratio of spike number induced by the second stimulation to the first stimulation. ***D***, ***E***, Expression of dominant-negative PKC-ε reverses the second stimulation-induced increase of the spike frequency. Dominant-negative PKC-ε completely reduces the increase in the number of amperometric spikes that have large peak amplitude (>20 pA), as well as small peak amplitude (<20 pA). ***F***, Knockdown of PKC-ε inhibits the increase in the number of large spikes. Distribution of large spikes represented as a percentage of spikes >20 pA peak amplitude among total amperometric spikes. Data presented in ***B***, ***C***, ***E***, and ***F*** are means ± SEM values. $^*p < 0.05$.

Table 1. Quantitative analysis of amperometric spikes

	1st stimulation[a]	2nd stimulation[b]
I_{max} (pA)	15.4 ± 7.4	24.8 ± 8.3***
$t_{1/2}$ (ms)[c]	37.6 ± 4.2	24.3 ± 5.0*
Quantal size (pC)	0.7 ± 0.2	1.0 ± 0.2*
Rise (pA/ms)	1.3 ± 0.4	2.0 ± 0.3**
Foot frequency (%)[d]	54.9 ± 13.0	58.1 ± 7.6
Foot I_{max} (pA)	0.3 ± 0.1	0.5 ± 0.2*
Foot charge (fC)	48 ± 22	103 ± 35*

The second stimulation-induced amperometric spikes were compared with the first stimulation-induced spikes. Data are shown as means ± SEM values. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

[a]One-hundred ninety-two and [b]260 amperometric spikes were averaged and analyzed by using a macro program (Mosharov and Sulzer, 2005).

[c]Half-width of spikes.

[d]Frequency of spikes having prespike foot among total amperometric spikes.

LDCV release via increasing the fusion frequency and quantal size.

PKC-ε regulates activity-induced translocation of vesicles

We reported previously that ADP is ascribed to activity-dependent increase of translocation of LDCVs (Park et al., 2006). To investigate whether PKC-ε is involved in activity-induced translocation of LDCVs, we performed electron microscopy analysis. Stimulation with DMPP increased the number of vesicles located near the plasma membrane within 500 nm from the plasma membrane (Fig. 4*A*,*B*). This enlargement of the vesicle pool size correlated with the increase in the number of amperometric spikes, suggesting that activity-induced vesicle translocation to the plasma membrane is associated with the increase in the number of fused vesicles (Figs. 2*C*, 3*B*). As shown in Figure 4*B*, inhibition of PKC by treatment with GF109203X abolished activity-induced vesicle translocation. Therefore, we suggest that PKC-ε regulates ADP of LDCV release via controlling activity-induced translocation of vesicles.

PKC-ε regulates MARCKS phosphorylation and F-actin disassembly

We next sought to identify the targets of PKC-ε. It has been shown that MARCKS can be directly phosphorylated by PKC, thereby inducing F-actin disassembly (Arbuzova et al., 2002). Our previous report also showed that F-actin disassembly was a

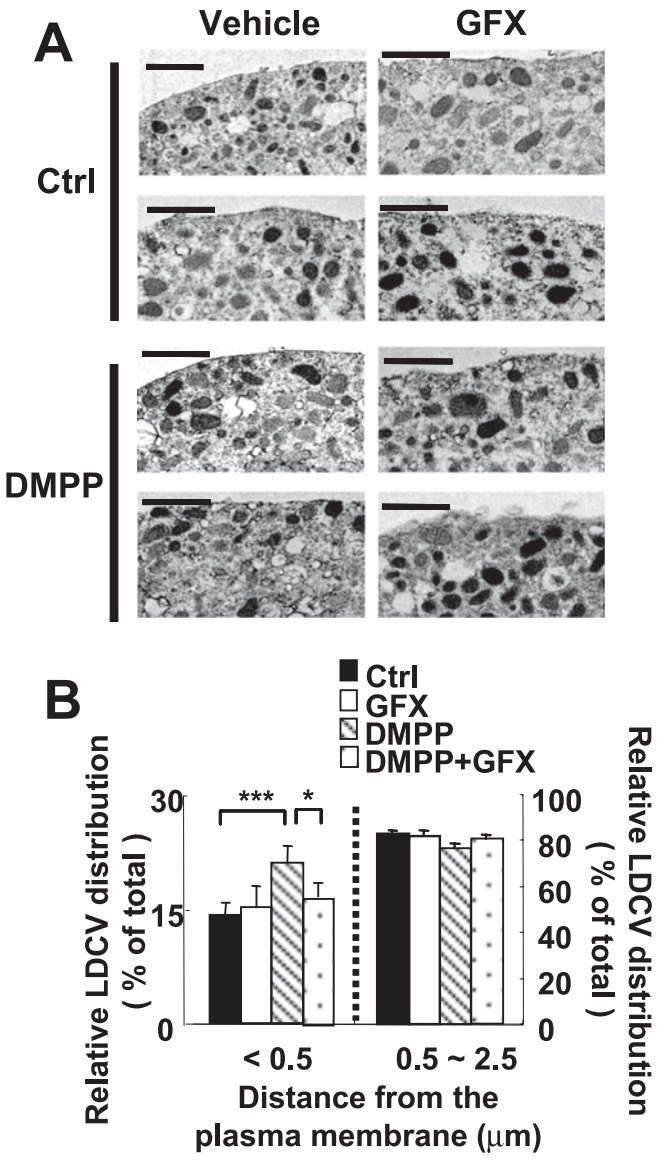

Figure 4. PKC-ε regulates activity-induced LDCV translocation. ***A***, nAChR stimulation increases the pool size of vesicles located near the plasma membrane. Cells were exposed to 10 μM DMPP for 1 min and then washed out for recovery. Shown are representative electron micrographs of control and DMPP-treated cells in the absence (vehicle) or presence of 1 μM GF109203X (GFX). Scale bar, 1 μm. ***B***, Distribution of LDCVs inside chromaffin cells after treatment with vehicle (833 vesicles in 10 cells), GFX alone (605 vesicles in 7 cells), or DMPP in the absence (807 vesicles in 10 cells) and presence (720 vesicles in 10 cells) of 1 μM GF109203X (GFX). Relative LDCV distribution is presented as a percentage of the number of LDCVs located from the plasma membrane to 0.5 and 0.5–2.5 μm inside. Data shown in ***B*** are means ± SEM values. $*p < 0.05$; $***p < 0.01$.

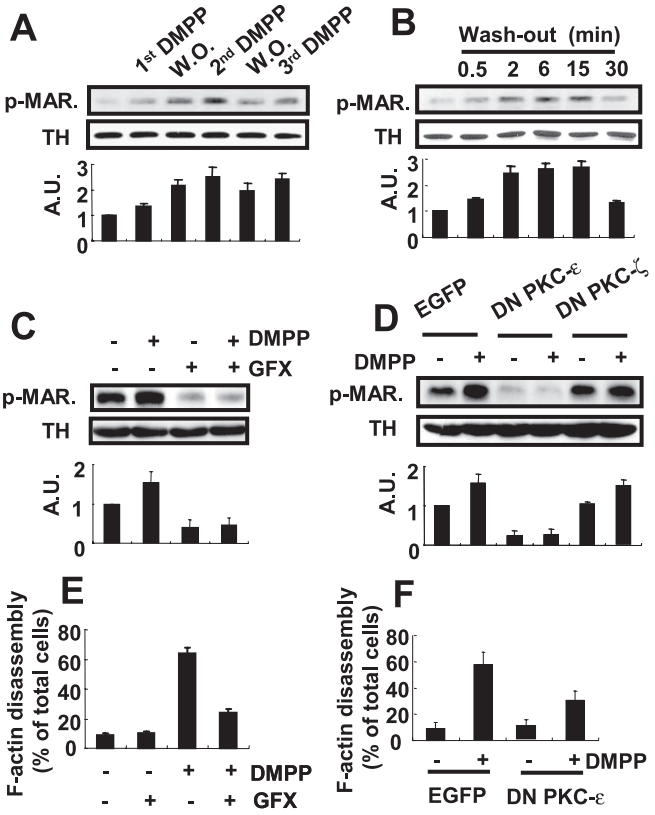

Figure 5. PKC-ε regulates MARCKS phosphorylation and F-actin disassembly. ***A***, Kinetics of phospho-MARCKS (p-MAR.) level during repetitive stimulation. After being repetitively stimulated with 10 μM DMPP as described in amperometry measurement (Fig. 1*A*), cells were subjected to SDS-PAGE. Cell lysates were immunoblotted with anti-phospho-MARCKS antibody. Immunoblotting with the TH antibody was performed for normalization. MARCKS phosphorylation was quantified by densitometry and normalized as an arbitrary unit (A.U.). W.O., Washout. ***B***, Activity-induced increase in phospho-MARCKS level. After stimulation with 10 μM DMPP for 20 s followed by washout for the indicated times, cell lysates were immunoblotted with anti-phospho-MARCKS antibody. ***C***, Inhibition of PKC-ε blocks activity-induced increase of phospho-MARCKS level. DMPP (10 μM, 1 min) was applied in the absence and presence of 1 μM GF109203X (GFX), followed by washout for 1 min. ***D***, Expression of dominant-negative PKC-ε inhibits activity-induced increase in phospho-MARCKS level. After being transfected with EGFP, dominant-negative PKC-ζ, or dominant-negative PKC-ε, cells were stimulated as previously indicated. ***E***, Treatment with GF109203X (GFX) inhibits F-actin disassembly induced by DMPP stimulation. Cells were examined for cortical fluorescence of rhodamine–phalloidin (indicating F-actin), and quantification of F-actin disassembly was presented as a percentage of cells having a fragmented cortical staining. ***F***, Dominant-negative PKC-ε blocks activity-induced F-actin disassembly. After being transfected with EGFP or dominant-negative PKC-ε, cells stimulated with DMPP were stained with rhodamine–phalloidin.

crucial step in ADP via controlling vesicle translocation (Park et al., 2006). We thus examined the level of phospho-MARCKS in response to repetitive stimulation. Phospho-MARCKS antibody that we used recognizes phospho-Ser152/156 residues, comprising the target site of PKC (Heemskerk et al., 1993). The level of phospho-MARCKS was elevated in response to the first stimulation, continued to increase, and was sustained during the second and the third stimulation (Fig. 5*A*). Interestingly, the kinetics of phosphorylation of MARCKS correlated with the window of time in which ADP occurred. We reported previously that exocytosis of LDCVs triggered by the second stimulation was strongly potentiated when interval time between the first and the second stimulation was 2–15 min, but the extent of ADP decreased when the interval time was longer than 30 min (Park et al., 2006). The level of phospho-MARCKS in response to 20 s of DMPP stimulation was also increased and sustained after 2 min and then decreased after 30 min (Fig. 5*B*). The similarity between time course of MARCKS phosphorylation and the extent of ADP suggests that MARCKS might be involved in ADP. Because PKC-ε regulates ADP, we tested whether phosphorylation of MARCKS was regulated by PKC-ε. Inhibition of PKC-ε by either treatment with GF109203X or expression of DN-PKC-ε reduced the level of MARCKS phosphorylation increased by nAChR activation. Basal level of MARCKS phosphorylation was also substantially blocked by inhibition of PKC-ε, suggesting that MARCKS is a downstream target of PKC-ε (Fig. 5*C*,*D*).

Stimulation of nAChR induces cortical F-actin disassembly (Rose et al., 2001), which is regulated by phosphorylation of

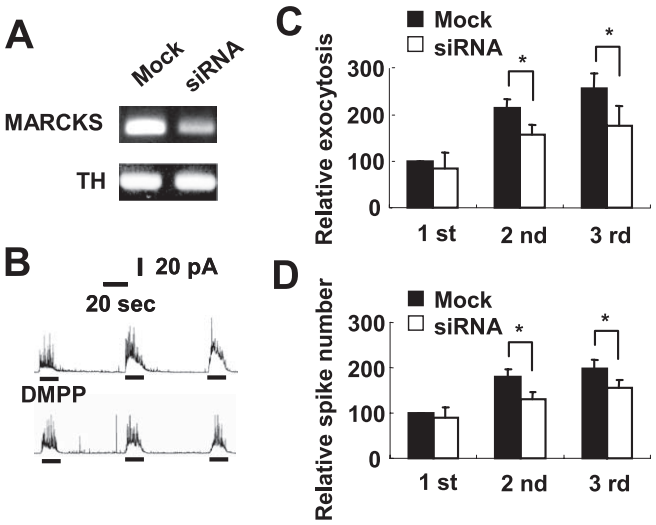

Figure 6. Knockdown of MARCKS attenuates ADP and decreases fused vesicle number. ***A***, Expression of MARCKS mRNA in the mock-transfected and MARCKS siRNA-transfected cells was analyzed by quantitative RT-PCR. TH expression is presented as a control for equal amount of cDNA. ***B***, Representative traces of amperometry are shown from cells transfected by the mock (top) or MARCKS siRNA (bottom) with EGFP-expressing plasmid. ***C***, Relative exocytosis of transfected cells with the mock ($n = 7$) and MARCKS siRNA ($n = 8$) was presented as a percentage of the first DMPP-induced catecholamine release obtained from the mock-transfected cells. ***D***, Expression of MARCKS siRNA reduces the spike number of amperometric current, which represents fused vesicles. Relative spike number is presented as a percentage of the first stimulation of the mock-transfected cells. Data presented in ***C*** and ***D*** are means ± SEM values. $^*p < 0.05$.

MARCKS (Arbuzova et al., 2002). We, therefore, examined whether PKC-ε mediated F-actin disassembly via phosphorylation of MARCKS. Either treatment with GF109203X or transfection with DN-PKC-ε resulted in inhibition of F-actin disassembly elicited by nAChR activation (Fig. 5*E*,*F*). Considering that F-actin disassembly is associated with vesicle mobilization and translocation from the reserve pool to the RRP (Vitale et al., 1995), these results suggest that PKC-ε regulates activity-induced vesicle translocation (Fig. 4) via modulating F-actin disassembly. Collectively, these data provide evidence that PKC-ε mediates ADP by means of regulating MARCKS phosphorylation, which induces F-actin disassembly.

Knockdown of MARCKS attenuates ADP

To further address the role of endogenous MARCKS in ADP of LDCV release, we used an RNA interference approach. Knockdown of MARCKS by transfection with specific siRNA resulted in attenuation of ADP compared with the level of scrambled siRNA-transfected cells (Fig. 6*C*). In addition, the number of amperometric spikes was smaller in cells expressing siRNA against MARCKS (Fig. 6*D*), showing that the number of fused vesicles was reduced. Together, these data suggest that MARCKS, a downstream target of PKC-ε, plays a role in ADP of LDCV release.

Discussion

In this study, we focused on the role of PKC-ε in ADP of exocytosis. Involvement of PKC in exocytosis has been investigated intensively by using phorbol 12,13-myristate acetate (PMA), an analog of DAG, as an activator of PKC. However, it has been reported that PMA has targets other than PKC to regulate exocytosis (Brose and Rosenmund, 2002; Rhee et al., 2002). Moreover, little is known about the isoform-specific functions of PKCs on exocytosis. Here we show that stimulation of nAChRs induces translocation and activation of PKC-ε (Fig. 1*C*) and suggest that PKC-ε mediates ADP by regulating F-actin disassembly. Transfection with DN-PKC-ε or siPKC-ε attenuated ADP without affecting the basal secretion (Figs. 2, 3). We provide evidence that PKC-ε mediates ADP via increasing the spike frequency and the quantal size of vesicles (Fig. 3), as well as the activity-induced vesicle translocation (Fig. 4). Moreover, DN-PKC-ε reduced the level of phospho-MARCKS and F-actin disassembly induced by nAChR activation (Fig. 5). Knockdown of MARCKS using siRNA inhibited ADP of LDCV release and reduced the number of vesicles, which are recruited and fused to the plasma membrane (Fig. 6). Altogether, we suggest that PKC-ε regulates ADP of LDCV release via phosphorylation of MARCKS, which induces F-actin disassembly and translocation of LDCVs.

It has been reported that depolarization of neuronal cells induces translocation and activation of PKC (Saitoh et al., 2001) and phosphorylation of substrates of PKC (Gianotti et al., 1992; Son et al., 1997), thereby regulating synaptic transmission (Brager et al., 2003). Electrical stimulation, which induces calcium influx, may cause translocation of PKC from the cytoplasm to the plasma membrane in which endogenous phospholipids can activate it (Akers and Routtenberg, 1987; Oancea et al., 1998). Although the most common lipid activator of PKC is diacylglycerol, many other endogenous phospholipids are also capable of activating PKC (Kaibuchi et al., 1981). Alternatively, the rise in calcium may activate phospholipase C (PLC) (Kim et al., 1999; Wang and Ashraf, 1999). In this mechanism, rises in calcium concentration lead to the generation of DAG, which then activates PKC. Calcium-induced activation of PLC has been described in several excitable tissues, including smooth muscle cells, synaptosomes, and cortical slices (Eberhard and Holz, 1987). We observed that nAChR stimulation results in translocation of PKC-ε to the membrane fraction, in which it can be readily activated.

Potentiation of exocytosis is usually attributed to the increase in the numbers of fused vesicles and/or the quantal size of neurotransmitters released from each vesicle. Repetitive stimulation of nAChRs increases the spike frequency, which represents enhanced number of vesicles fused to the plasma membrane (Fig. 3). Also, repetitive stimulation of nAChR increases the quantal size via modulating fusion pore kinetics (Table 1). The number of vesicles that have large peak amplitudes (>20 pA) dramatically increased compared with those with small peak amplitudes (<20 pA) (Fig. 3*C*). Inhibition of PKC-ε by either expression of DN-PKC-ε or siPKC-ε attenuated the increase in both the spike frequency and the quantal size (Fig. 3*D–F*).

MARCKS, which is known to regulate F-actin dynamics, has been reported to be phosphorylated by many kinases, such as mitogen-activated protein kinases, cyclin-dependent kinases, PKA, Rho kinase, as well as PKC (Arbuzova et al., 2002; Sasaki, 2003). Phospho-MARCKS antibody that we used in the present study recognizes residues phosphorylated by PKC (Heemskerk et al., 1993). Because inhibition of PKC-ε by either treatment with GF109203X or expression of DN-PKC-ε reduced the level of phospho-MARCKS (Fig. 5), we suggest that MARCKS is a downstream target molecule of PKC-ε. We also showed that inhibition of PKC-ε reduced F-actin disassembly (Fig. 5*E*,*F*), which was proven previously to regulate vesicle translocation (Rose et al., 2001). Knockdown of MARCKS resulted in the attenuation of ADP of LDCV release and reduction in the number of vesicles (Fig. 6), suggesting that MARCKS is involved in ADP as a downstream molecule of PKC-ε.

Chromaffin cells are stimulated by acetylcholine, which is re-

petitively secreted from preganglionic sympathetic neurons, resulting in catecholamine release into the bloodstream. Catecholamine released from chromaffin cells mediates the "fight-or-flight" response as a defense mechanism against the environment (Henry, 1992). Additionally, this system is also activated by a social and/or mental stress. The level of catecholamine in the blood is elevated within minutes in response to an acute stressor and sustained depending on the mental and physical load (Forsman and Lindblad, 1983). Repetitive stress at work gives rise to chronically elevated levels of catecholamine, probably causing health problems (Lundberg et al., 1999). Chromaffin cells show ADP of LDCV release only during repetitive nAChR stimulation, in contrast to excitable secretory cells that usually deplete the pool of the RRP during repetitive stimulation. Therefore, we suggest that ADP of LDCV release might be a physiological mechanism to keep elevated levels of catecholamine during activation of the sympathetic nervous system.

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