Biochemical and Biophysical Research Communications 432 (2013) 384–388

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Both C1B domain and pseudosubstrate region are necessary for saturated fatty acid-induced translocation of εPKC to the plasma membrane: Distinct role of intramolecular domains for different translocation

Takaaki Nishimoto [a,1], Kaori Kashiwagi [a], Naoaki Saito [a,*], Yasuhito Shirai [a,b,*]

[a] The Laboratory of Molecular Pharmacology, Biosignal Research Center, Kobe University, Rokkodai-cho 1-1, Nada-ku, Kobe 657-8501, Japan
[b] Laboratory of Chemistry & Utilization of Animal Production Resources, Department of Applied Chemistry in Bioscience, Graduate School of Agricultural Science, Kobe University, Rokkodai-cho 1-1, Nada-ku, Kobe 657-8501, Japan

ARTICLE INFO

Article history:
Received 9 January 2013
Available online 24 January 2013

Keywords:
Protein kinase C
Saturated fatty acid
Tridecanoic acid
Intramolecular domains
Mechanism of translocation

ABSTRACT

It is well known that protein kinase C (PKC) shows different translocation depending on subtype and stimulation, contributing to the physiological importance of the enzyme. However, molecular mechanism causing the different translocation has been unknown. Therefore, using GFP-tagged mutant εPKC, we attempted to identify the intramolecular domains required for saturated fatty acid-induced translocation of εPKC to the plasma membrane, and compared with those necessary for unsaturated fatty acid-induced translocation to the Golgi complex.

We found that, unlike in the case of unsaturated fatty-acid induced translocation, both C1B domain and pseudosubstrate region are necessary for the saturated fatty acid-induced translocation of εPKC to the plasma membrane. The results suggest that different domains of PKC mediate distinct translocation depending on different stimulations, contributing to their subtype- and stimulation-specific functions.

1. Introduction

Protein kinase C (PKC), was first described as a serine/threonine kinase by Nishizuka and colleagues [1], and consists of at least 11 subtypes that are classified into three subfamilies based on their structural difference and requirement for activators: classical PKCs (PKCα, βI, βII and γ), novel PKCs (PKCδ, ε, η and θ), and atypical PKCs (PKCζ and λ/ι) [2].

PKC plays a key role in cellular responses such as neurotransmission, gene expression, and cell growth and differentiation [3,4]. All PKCs possess an amino-terminal regulatory domain and a catalytic domain in the carboxyl terminus. The regulatory domain of classical PKCs (cPKC) contains a variable region 1 (V1), a pseudosubstrate motif (PS), and a conserved region 1 and 2 (C1 and C2). The C1 domain has two cysteine-rich loops (C1A and C1B) that are the binding sites for diacylglycerol (DG) and/or phorbol esters [5,6], while the C2 region binds to Ca^{2+}[6]. Novel and atypical PKCs (nPKCs and aPKCs) lack the C2 domain [7–9] and aPKCs have only one cysteine-rich loop in the C1 region [7,9,10]. Thus, nPKC and aPKC are not activated by Ca^{2+} and aPKCs do not depend on DG. These differences in the structure and enzymatic properties strongly suggest specific functions of each subtypes .

In addition to the subtype-specific activators, each PKC subtype has spatially and temporally different targeting sites depending on stimulations [11–15]. For example, unsaturated fatty acid including arachidonic acid (AA) induces rather slow and irreversible translocation of εPKC from the cytoplasm to the Golgi complex, while saturated fatty acids including tridecanoic acid (TDA) and palmitic acid induce rapid and reversible translocation of εPKC to the plasma membrane [16,17]. On the other hand, δPKC cannot respond to any fatty acids [18]. These results suggest that the stimulation-dependent targeting site is important for the distinct functions of PKC. However, the molecular mechanism to cause the different translocation has not been clear.

Therefore, in a series of the experiments to investigate the molecular mechanisms for the distinct translocation of PKCs, we attempted to identify the intramolecular domains responsible for the saturated fatty acid-induced translocation of εPKC to the plasma membrane, and compared with the domain(s) required for the unsaturated fatty acid-induced translocation to the Golgi complex.

* Corresponding authors. Address: Laboratory of Chemistry & Utilization of Animal Production Resources, Department of Applied Chemistry in Bioscience, Graduate School of Agricultural Science, Kobe University, Rokkodai-cho 1-1, Nada-ku, Kobe 657-8501, Japan (Y. Shirai).

E-mail addresses: naosaito@kobe-u.ac.jp (N. Saito), shirai@kobe-u.ac.jp (Y. Shirai).

[1] Present address: Department of Physiology, Division of Bioinformation, Hyogo College of Medicine, Mukogawa-cho 1-1, Nishinomiya 663-8501, Japan.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.01.048

2. Experimental procedures

2.1. Materials

Tridecanoic acid and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) were purchased from Sigma. FuGENE™ 6 Transfection Reagent was obtained from Roche Molecular Biochemicals. All the other chemicals used were of analytical grade.

2.2. Cell culture

COS7 and HEK293 cells were purchased from the Riken cell bank (Tsukuba, Japan). Both of the cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY) at 37 °C in a humidified atmosphere containing 5% CO_2. The media containing 25 mM glucose were buffered with 44 mM $NaHCO_3$ and were supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). The fetal bovine serum used was not heat-inactivated. For transfection experiments, HEK293 cells were trypsinized and seeded at a density of 1×10^5 cells/35-mm on glass-bottomed culture dishes (MatTek Corp., Ashland, MA) and incubated for 16–24 h before transfection.

2.3. Transfection of the GFP-tagged PKCs and mutants

HEK293 cells spread on 3.5 cm glass-bottomed dish (MatTek Corp.) were transfected using 3 μl of FuGENE™ 6 Transfection Regent and 1 μg of DNA according to the manufacturer's protocol. Transfected cells were cultured at 37 °C for 16–48 h prior for imaging.

2.4. Construction of plasmids encoding the GFP-tagged PKCs and mutants

The constructs encoding GFP-conjugated rat δPKC (δPKC) and εPKC (εPKC) were previously described [14,16]. The cDNA encoding the various deletion mutants of εPKC and chimeras of εPKC containing δC1B (ε(δC1B)) and δPKC containing εC1B (δ(εC1B)) were generated as previously described [18]. The chimera of δ-PKC containing εV1, PS, C1B (δ(εV1, PS, C1B)) was produced by PCR using two plasmids as templates at one reaction. We used BS789 (rat δ-PKC containing εC1B in pCR™2.1) and BS797 (rat ε-PKC containing δC1B in pCR™2.1) [18] as the templates with δF475 (CACGAGTTCATCGCCACC)/εR507 (GCCGTTAACCTGGTGGAC) as the primers using the ExSite™ PCR-based Site-directed Mutagenesis kit.

2.5. Immunoblotting for GFP-tagged PKCs

COS7 cells were transiently transfected by electroporation and cultured for 2 days. The transfected cells were harvested with phosphate-buffered saline (PBS) and concentrated by centrifugation. The pellet was resuspended in 200 μl of homogenization buffer containing 1% Triton X-100 (250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 20 mM Tris–HCl, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) and homogenized by sonication. After centrifugation, 20 μg of protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 7.5% polyacrylamide gel, and transferred to polyvinylidiene difluoride filters (Millipore, Bedford, MA). Nonspecific binding sites were blocked with 5% skim milk in 0.01 M PBS containing 0.03% Triton X-100 (PBS-T) (18 h, at 4 °C). The blots were probed with anti-GFP polyclonal antibody (CLONTECH Laboratories, Inc., Palo Alto, CA) (diluted 1:1000) for 1 h at 25 °C. After washing with PBS-T, the blots were incubated with peroxidase-conjugated or anti-rabbit IgG (1 h, at 25 °C). The immunoreactive bands were visualized with an enhanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, England).

2.6. Confocal microscopy

HEK293 cells transfected with the GFP-tagged PKCs and mutants were cultured for 16–48 h for maximal GFP expression. The media was then replaced with Ringer's solution composed of 135 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, and 10 mM glucose, pH 7.3. Translocation of the GFP-tagged mutant PKCs was triggered by the addition of the various stimuli to the Ringer's solution to obtain the appropriate final concentration. All experiments were done at 37 °C. The GFP fluorescence was monitored by confocal laser scanning fluorescent microscopy (Carl Zeiss, Jena, Germany) at 488-nm argon excitation with a 515-nm long pass barrier filter. Time series images were recorded before and after stimulation.

3. Results

3.1. PS is indispensable for the saturated fatty acid-induced translocation of εPKC to the plasma membrane

In resting HEK293 cells, full-length εPKC-GFP (εPKC) was expressed throughout the cytoplasm, but it was excluded from the nuclei (Fig. 1, top left). After stimulation with tridecanoic acid (TDA) at 100 μM, εPKC was translocated from the cytoplasm to the plasma membrane within 20 s (Fig. 1, top center), and then it was re-translocated to the cytoplasm within 3 min (Fig. 1, top right). To identify the domains required for the TDA-induced membrane translocation of εPKC, we first used a series of GFP-tagged N-terminus deletion mutants of εPKC. Appropriate molecular weight of each GFP-tagged mutants and no significant degradated products were confirmed [18]. All deletion mutants except for ΔV1-PS were localized in the cytoplasm before the stimulation, similar to the intracellular localization of full-length ε-PKC. ΔV1-PS was localized heterogeneously in the cytoplasm. When stimulated with 100 μM TDA, ΔV1 showed the same translocation pattern and time course as full-length εPKC, indicating that V1 region is not necessary for the TDA-induced translocation of εPKC. In contrast, further deletion of psudosubstrate region (PS) abolished the translocation (Fig. 1, third row). However, ΔV1-PS did not lost the ability to translocate, because it showed the translocation to plasma membrane in response to phorbol 12-myristate 13-acetate (TPA) (Table 1). In addition, TDA did not changed distributions of ΔV1-PS-C1A and ΔV1-PS-C1 (Fig. 1, forth and bottom row). These results suggest an important role of PS in the TDA-induced membrane translocation of εPKC. To confirm the importance of PS, we further used a mutant lacking only PS (ΔPS). ΔPS was localized heterogeneously in the cytoplasm before stimulation, and did not respond to TDA (Fig. 2. top), confirming the necessity of PS for the TDA-induced translocation of εPKC. In a case of palmitic acids at 100 μM, all mutants showed similar translocation pattern to those by TDA (data not shown). These results indicate that PS region is necessary for the saturated fatty acid-induced transient translocation of εPKC to the plasma membrane.

3.2. C1B is also involved in the saturated fatty acid-induced translocation of εPKC

Previously, we found that C1B region is critical for the AA-induced translocation of εPKC to the Golgi complex [18]. Therefore, to check the importance of C1B in the fatty acid-induced translocation to the plasma membrane, we tested effect of TDA on the

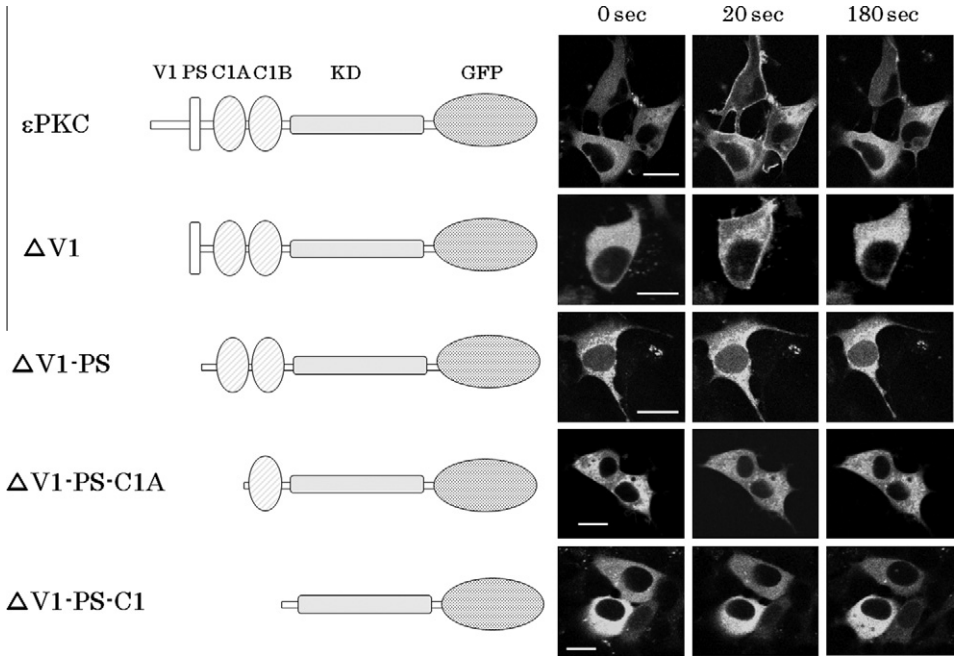

Fig. 1. Effects of tridecanoic acid on the translocation of N-terminus deletion mutants of εPKC. Various N-terminal deletion mutants of εPKC-GFP were expressed in HEK293 cells, respectively. Live cell images were taken as indicated times after stimulation by tridecanoic acid (100 μM). Bars, 10 μm.

Table 1
Diversity in translocation of εPKC deletion mutants and chimeras of εPKC and δPKC. "+" and "−" reflect translocation and no translocation, respectively. N.T. means "not tested".

	TDA	AA	TPA
Wild type ε-PKC	+	+	+
ΔV1	+	+	+
ΔV1-PS	−	+	+
ΔV1-PS-C1A	−	+	+
ΔV1-PS-C1A, B	−	−	−
ΔPS	−	+	+
ΔC1A	+	+	+
ΔC1B	−	−	+
δ(εV1,PS,C1B)	+	N.T	+
δ(εC1B)	+	+	+
ε(δC1B)	−	−	+
Wild type δ-PKC	−	−	+

distribution of a mutant lacking C1B (ΔC1B). ΔC1B was localized throughout the cytoplasm before stimulation, similar to full-length ε-PKC (Fig. 2). However, TDA did not induced rapid and reversible translocation of ΔC1B to the plasma membrane. In contrast, ΔC1A, which lacks C1A domain, showed similar translocation to that of εPKC in response to TDA (Fig. 2). Importantly, both ΔC1A and ΔC1B were translocated to the plasma membrane in response to TPA (Table 1), indicating that not only PS but also C1B domain is involved in the TDA-induced translocation of ε-PKC. In the case of palmitic acid stimulation, similar results were obtained.

3.3. PS of εPKC, but not C1B domain, is convertible to that of δPKC in the saturated fatty acid-induced translocation

There is a risk that the deletion mutants used in Figs. 1 and 2 may not form original three-dimensional structure of full-length εPKC, because of the lack of some domains. Therefore, to further confirm the involvement of PS and C1B domain in the saturated fatty acid-induced translocation of εPKC, we used the chimeras of εPKC and δPKC, as saturated fatty acids do not induce translocation of δPKC [11].

δ(εC1B) which is δ-PKC having εPKC C1B domain and ε(δC1B) which is εPKC having δPKC C1B domain were previously described [18]. In addition to the constructs, we made an additional chimera δ(εV1, PS, C1B), which is δ-PKC having V1, PS and C1B regions of εPKC. Appropriate molecular weight and no degradation products of δ(εV1, PS, C1B) were confirmed (data not shown). In resting cells, δ(εV1, PS, C1B) was expressed throughout in the cytoplasm with slight accumulation in the perinuclear region (Fig. 3, top row). TDA at 100 μM induced translocation of δ(εV1, PS, C1B) to the plasma membrane, although it did not come back to the cytoplasm (Fig. 3, top row), indicating that both PS and C1B domains of εPKC are sufficient for the TDA-induced membrane translocation. In contrast, ε(δC1B) was localized heterogeneously in the cytoplasm and it did not significantly change the distribution in response to TDA (Fig. 3, bottom row), confirming that importance of C1B domain for the TDA-induced translocation of εPKC. Surprisingly, δ(εC1B) showed similar translocation pattern and time course to that of full-length εPKC (Fig. 3, middle row), suggesting that εPKC PS domain is convertible to that of δPKC.

In conclusion, both PS and C1B domains are important for the saturated fatty acid-induced translocation of εPKC to the plasma membrane. Especially, C1B domain is indispensable for subtype-specific function of εPKC.

4. Discussion

Recent studies on PKC translocation using GFP-fusion protein revealed that each PKC subtype has a unique targeting site that depends on various stimulations. Specifically, targeting of εPKC by fatty acid is diverse. Previously, we reported that effects of 11 fatty acids including TDA and AA on the translocation of εPKC [16], and showed the remarkable translocation of εPKC by various fatty acids. For example, saturated fatty acids including TDA induce a rapid and transient translocation of εPKC to the plasma membrane, while unsaturated fatty acids including AA evoke a rather slower translocation to the Golgi complex [16]. Importantly, the fatty acid-dependent translocation was observed in the purinergic receptor stimulation [17], and these saturated and unsaturated

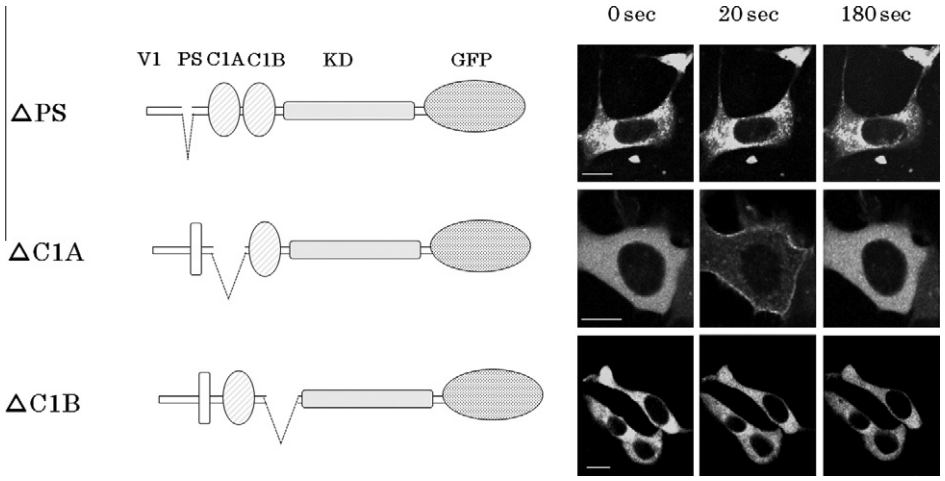

Fig. 2. Effects of tridecanoic acid on the translocation of deletion mutants of each domain of εPKC. Deletion mutants, which is deleted each domain of εPKC-GFP, were expressed in HEK293 cells, respectively. Live cell images were taken as indicated times after stimulation by tridecanoic acid (100 μM). Bars, 10 μm.

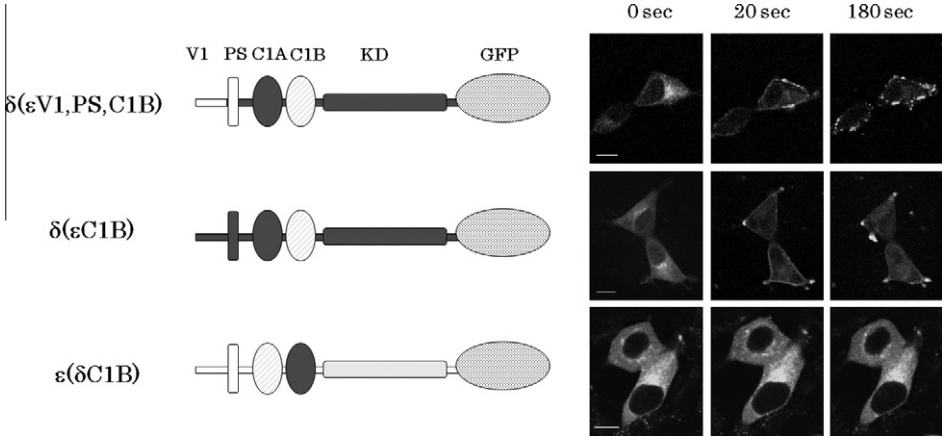

Fig. 3. Effects of tridecanoic acid on the translocation of εPKC and δPKC chimeras. δ (εV1, PS, C1B) is the mutant of δPKC having the V1, PS and C1B domain of εPKC. δ (εC1B) is the mutant of δPKC having the C1B domain of εPKC. ε (δC1B) is the mutant of εPKC having the C1B domain of δPKC. Cells expressing these chimers were stimulated by tridecanoic acid (100 μM). Live cell images were taken as indicated times after stimulation by tridecanoic acid. Bars, 10 μm.

fatty acids potentiated the activity of εPKC [3,12,19]. These facts suggest that diversity of translocation contributes to physiological functions of εPKC.

Similarly, different translocation of δPKC by phorbol esters and related compounds were reported [20]. Two tumor promoters, TPA and 12-deoxyphorbol 13-monoesters, induced plasma membrane translocation of δ-PKC followed by slower nuclear translocation. In contrast, only nuclear translocation of δ-PKC was induced by similar δPKC activators, bryostatin and 12-deoxyphorbol 13-phenylacetate that have anti-tumor promoting effects [20]. These results suggest that distinct pharmacological effects of the drugs may be exerted due to the different translocation of the PKC subtype.

However, molecular mechanism causing the different translocation had been unknown. In this study, we showed for the first time that PS, in addition to C1B domain, region is important for the TDA-induced translocation of εPKC to the plasma membrane. On the other hand, we have previously revealed that only C1B domain is indispensable for the AA-induced translocation of εPKC to the Golgi complex ([18] and Table 1). The results implicate that different intramolecular domains have distinct roles in the respective translocations. To speculate the function of each domain, we investigated localization of PS or C1B domains of εPKC. V1-PS was localized on the plasma membrane, while C1B accumulated in the perinuclear region (Fig. 4). Based on the localization and the results summarized in Table 1, we hypothesize that, in a case of fatty acid-induced translocation, TDA interacts with C1B domain, resulting in

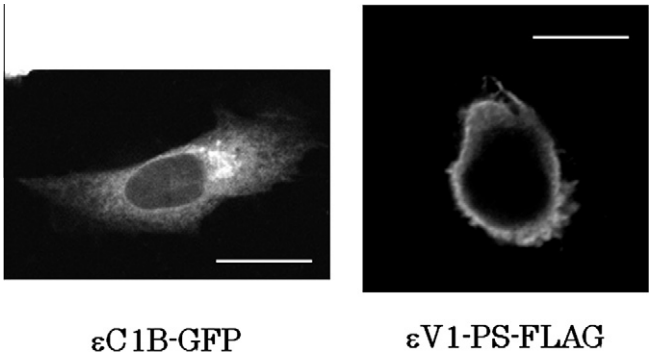

Fig. 4. Localization of V1-PS and C1B domains of εPKC. (A) Localization of C1B domain of εPKC. GFP-fused C1B domain of εPKC was expressed in HEK293 cells and the image was taken by confocal microscope. Bar, 10 μm. (B) Localization of V1-PS domain of εPKC. FLAG-fused V1-PS domain of εPKC was expressed in HEK293 cells. After fixation, the localization was visualized by mouse FLAG (SIGMA) and Texas-red conjugated anti-mouse antibodies. Bar, 10 μm.

conformational change. Finally, εPKC binds to the plasma membrane through PS region. In the case of unsaturated fatty acid-induced translocation, AA interacts with C1B domain and εPKC binds to the Golgi complex through the C1B domain. In other words, PS region is necessary for binding of εPKC to the plasma membrane, while C1B is important for fatty acid binding itself. Indeed, the εPKC mutants lacking C1B including ΔC1B and ΔV1-PS-C1A, C1B lost the ability to respond to any fatty acids (Table 1). In addition to specific domain, they may also include differences in phosphorylation, interaction partners and/or specific conformation changes of PKC. Further experiments are necessary.

In conclusion, saturated fatty acids induce the translocation of εPKC to the plasma membrane via C1B domain and PS region, indicating that different domains of PKC mediate translocation in response to different stimulations. The present study is an important step to comprehensively understand the molecular mechanism of PKC translocation contributing to their subtype- and stimulation-specific functions.

References

[1] M. Inoue, A. Kishimoto, Y. Takai, Y. Nishizuka, Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain, J. Biol. Chem. 252 (1977) 7610–7616.
[2] S.F. Steinberg, Structural basis of protein kinase C isoform function, Physiol. Rev. 88 (2008) 1341–1378.
[3] Y. Nishizuka, The role of protein kinase C in cell surface signal transduction and tumour promotion, Nature 308 (1984) 693–698.
[4] Y. Nishizuka, Protein kinase C and lipid signaling for sustained cellular responses, FASEB J. 9 (1995) 484–496.
[5] Y. Nishizuka, The molecular heterogeneity of protein kinase C and its implications for cellular regulation, Nature 334 (1988) 661–665.
[6] Y. Ono, T. Fujii, K. Igarashi, T. Kuno, C. Tanaka, U. Kikkawa, Y. Nishizuka, Phorbol ester binding to protein kinase C requires a cysteine-rich zinc-finger-like sequence, Proc. Natl. Acad. Sci. USA 86 (1989) 4868–4871.
[7] P.J. Parker, L.V. Dekker, Introduction, in: P.J. Parker, L.V. Dekkers (Eds.), Protein Kinase C, RG Landes, Austin, 1997, pp. 1–9.
[8] H. Hug, T.F. Sarre, Protein kinase C isozymes: divergence in signal transduction?, Biochem J. 291 (1993) 329–343.
[9] K. Akimoto, K. Mizuno, S. Osada, S. Hirai, S. Tanuma, K. Suzuki, S. Ohno, A new member of the third class in the protein kinase C family, PKC lambda, expressed dominantly in an undifferentiated mouse embryonal carcinoma cell line and also in many tissues and cells, J. Biol. Chem. 269 (1994) 12677–12683.
[10] L.A. Selbie, C. Schmitz-Peiffer, Y. Sheng, T.J. Biden, Molecular cloning and characterization of PKC iota, an atypical isoform of protein kinase C derived from insulin-secreting cells, J. Biol. Chem. 268 (1993) 24296–24302.
[11] Y. Shirai, N. Sakai, N. Saito, Subspecies-specific targeting mechanism of protein kinase C, Jpn. J. Pharmacol. 78 (1998) 411–417.
[12] K. Kasahata, U. Kikkawa, Distinct effects of saturated fatty acids on protein kinase C subspecies, J. Biochem. 117 (1995) 648–653.
[13] N. Sakai, K. Sasaki, N. Ikegaki, Y. Shirai, Y. Ono, N. Saito, Direct visualization of the translocation of the γ-subspecies of protein kinase C in living cells using fusion proteins with green fluorescent protein, J. Cell Biol. 139 (1997) 1465–1476.
[14] S. Ohmori, Y. Shirai, N. Sakai, M. Fujii, H. Konishi, U. Kikkawa, N. Saito, Three distinct mechanism for translocation and activation of the δ subspecies of protein kinase C, Mol. Cell. Biol. 18 (1998) 5263–5271.
[15] T. Kajimoito, S. Ohmori, Y. Shirai, N. Sakai, N. Saito, Subtype-specific translocation of the δ subtype of protein kinase C and its activation by tyrosine Phosphorylation induced by ceramide in HeLa cells, Mol. Cell. Biol. 21 (2001) 1769–1783.
[16] Y. Shirai, K. Kashiwagi, K. Yagi, N. Sakai, N. Saito, Distinct effects of fatty acids on translocation of gamma- and epsilon-subspecies of protein kinase C, J. Cell Biol. 143 (1998) 511–521.
[17] Y. Shirai, K. Kashiwagi, N. Sakai, N. Saito, Phospholipase A_2 and its products are involved in the purinergic receptor-mediated translocation of protein kinase C in CHO-K1 cells, J. Cell Sci. 113 (2000) 1335–1343.
[18] K. Kashiwagi, Y. Shirai, M. Kuriyama, N. Sakai, N. Saito, Importance of C1B domain for lipid messenger-induced targeting of protein kinase C, J. Biol. Chem. 277 (2002) 18037–18045.
[19] L.C. McPhail, C.C. Clayton, R. Snyderman, A potential second messenger role for unsaturated fatty acids: activation of Ca^{2+}-dependent protein kinase, Science 224 (4649) (1984) 622–625.
[20] Q.J. Wang, D. Bhattacharyya, S. Garfild, K. Narco, V.E. Marquez, P.M. Blunberg, Differential localization of protein kinase C delta by phorbol esters and related compounds using a fusion protein with green fluorescent protein, J. Biol. Chem. 274 (1999) 37233–37239.