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Full paper

Propofol induced diverse and subtype-specific translocation of PKC families

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ABSTRACT

Propofol is the most commonly used anesthetic. Immunohistochemical studies have reported that propofol translocated protein kinase Cs (PKCs) in cardiomyocyte in a subtype-specific manner; however detailed features of the propofol-induced translocation of PKCs remain unknown. In this study, we performed real-time observation of propofol-induced PKC translocation in SH-SY5Y cells expressing PKCs fused with a fluorescent protein. Propofol unidirectionally translocated γPKC-GFP, a conventional PKC, and ζPKC-GFP, an atypical PKC, to the plasma membrane and nucleus, respectively, whereas the propofol-induced translocation of novel PKCs was diverse and subtype-specific among δPKC, εPKC and ηPKC. The propofol-induced translocation of εPKC-GFP was especially complicated and diverse, that is, 200 μM propofol first translocated εPKC-GFP to the perinuclear region. Thereafter, εPKC was translocated to the nucleus, followed by translocation to the plasma membrane. Analysis using a mutant εPKC in which the C1 domain was deleted demonstrated that the C1b domain of εPKC was indispensable for its translocation to the perinuclear region and plasma membrane, but not for its nuclear translocation. An in vitro kinase assay revealed that propofol increased the activities of the PKCs activities at the concentration that triggered the translocation. These results suggest that propofol could translocate PKCs to their appropriate target sites in a subtype-specific manner and concomitantly activated PKCs at these sites, contributing to its beneficial or adverse effects.

1. Introduction

Protein kinase C (PKC), a family of serine/threonine kinase, is involved in diverse cellular functions.[1,2] In the central nervous system, PKC plays an important role in neurotransmission by regulating neurotransmitter release and modifying functions of neurotransmitter receptors and ion channels.[2,3]

Among the PKC family, there are more than ten subtypes, which are calcified into three groups, conventional PKCs (cPKCs), novel PKCs (nPKCs) and atypical PKCs (aPKCs), based on the structure of their regulatory domain (Fig. 1).[1,4]

PKC consists of two common domains, a kinase domain and a regulatory domain. The kinase domain functions as a kinase itself, and the regulatory domains accept various stimulants. As a regulatory domain, the C1 domain is known to respond to phospholipids, and the C2 domain binds to calcium ions. The C1 domain is subdivided into two regions, the C1a and C1b domains.

cPKCs have both a C1 and C2 domain; therefore they are activated by membrane-derived phospholipids and Ca^{2+}, which is mobilized into the cytosol from the endoplasmic reticulum (ER).

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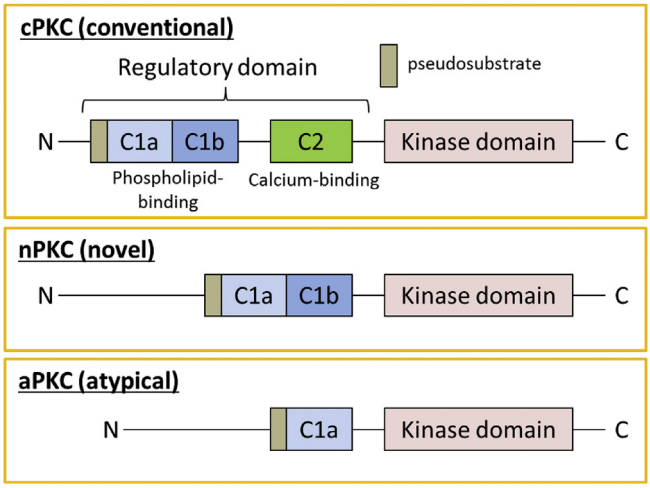

Fig. 1. Structure of PKC families.

nPKCs do not require Ca^{2+} for their activation because nPKCs do not contain a C2 domian.[5] aPKCs only have one C1 subdomain, and Ca^{2+} is not necessary for their activation.[6,7]

The cellular localization of PKCs is altered upon its activation, the phenomenon of which is called "PKC translocation".[8–10] For instance, cPKCs are transiently translocated from the cytosol to the plasma membrane upon the stimulation of G-protein-coupled receptors (GPCRs).[9,10]

In addition to GPCR stimulation, various lipids mediators can trigger PKC translocation, the target sites of which depend on the lipid mediators and the PKC subtypes.[11]{ For example, arachidonic acids, one of the lipid mediators, translocate γPKC to the plasma membrane, εPKC to the Golgi apparatus and ζPKC to the nucleus.[10–12] Ceramide, another lipid mediator produced by sphingomyelinase activation, also translocates δPKC to the Golgi apparatus.[13] These findings lead to the hypothesis that the function of PKC depends on the location of its translocation and what types of substrates the PKC phosphorylates at the translocated sites in case of its activation.[9,10] For this reason, PKC translocation is a very important phenomenon when considering the role of PKC.

Propofol was discovered and clinically applied to anesthesia in 1977 by Kay and Rolly.[14] At present, this anesthetic agent is most commonly used because of its rapid anesthetic induction and reversal. Propofol has been considered to exert its anesthetic effects by binding to the $GABA_A$ receptor, thereby, enhancing the inhibitory effects of GABA on the central nervous system.[15] Regarding the involvement of PKC in the action of propofol, this anesthetic is known to dose-dependently enhance the activity of purified PKC in vitro.[16] Additionally, propofol has been reported to exert its protective effects on ischemia and reperfusion damage via the activation of PKC in the heart.[17,18] Immunoblotting and immunocytochemistry have revealed that propofol translocates various types of PKC to the subcellular region in a subtype-specific manner in cardiomyocytes.[19]

Thus, PKC is hypothesized to be implicated in the exertion of various effects of propofol; however, how propofol induces PKC translocation and activation remains unclear. In this study, we aimed to elucidate the effects of propofol on the translocation of PKC and enzymatic activity. For this purpose, we performed the following studies: 1) an analysis of propofol-induced PKC translocation using PKC-GFP, 2) an examination of the significance of the C1 domain in propofol-induced PKC translocation, and 3) an investigation of the mechanism by which propofol affects PKC activity as a kinase in vitro.

2. Materials and methods

2.1. Materials

Propofol was obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). 12-O-Tetradecanoylphorbol 13-acetate (TPA) was purchased from Sigma (St. Louis, MO, USA). [^{32}P]γATP and Protein A Sepharose 4-fast flow were purchased from GE Health Care (Little Chalfont, UK). All of the expression plasmids for PKC-GFPs or mutant PKC-GFP, which have been described throughout the literature,[8,11–13,20] and the polyclonal anti-GFP antibody for immunoprecipitation were purchased from Dr. Saito (Biosignal Research Center, Kobe University). Glass-bottom culture dishes were purchased from MatTek Corporation (Ashland, OR, USA). All of the other chemicals used were of analytical grade.

2.2. Construction of adenovirus vectors for the expression of PKC-GFPs or δPKC-DsRed

To construct adenovirus vectors that can express PKC-GFPs or δPKC-DsRed, we used the pAdEasy system (Agilent Technology, Santa Clara, CA, USA). Briefly, cDNAs for PKCs except δPKC were first inserted into the pEGFP-N1 (Clontech Takara, Japan). Thereafter, these PKC cDNAs combined with EGFP were inserted into the pShuttle-CMV vector. δPKC cDNA was inserted into the pDsRed monomer-N1 (Clontech Takara, Japan), and a δPKC-DsRed fragment was inserted into the pShuttle-CMV. These shuttle vectors were recombined with pAdEasy-1, an adenoviral backbone cosmid vector, in the BJ5183 *E. coli* strain. The recombinant adenoviral genome was digested from the cosmid vector by PacI and transfected into HEK293 cells, which stably expressed the E1 gene and produced the E1 gene-deleted adenoviral vector. Proliferated adenoviral vectors were extracted from HEK293 cells and concentrated by cesium chloride ultracentrifugation. The αPKC, γPKC and δPKC cDNAs originated from human, and the εPKC, ηPKC and ζPKC cDNAs were from rat.

2.3. Construction of plasmids encoding the deletion mutants of εPKC-GFP or γPKC-GFP

The plasmids encoding the deletion mutants of εPKC-GFP were generated as previously described.[12] The constructs encoding GFP-fused rat εPKC were previously described.[11] Briefly, the cDNA encoding deletion mutants of εPKC were generated by PCR using BS 495 (rat εPKC in pCRTM2.1)[11] as the template. The cDNAs encoding domain-deleted εPKCs were produced using the ExSiteTM PCR-based Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) with BS495 as a template. The PCR products for deletion mutants of εPKC were subcloned into the EGFP expression vector (BS340). The plasmids encoding mutant γPKC, the lipid-binding sites in the C1a and C1b domains of which were lost (non-lipid-biding γPKC) were produced as previously described.[4,8]

2.4. Cell culture and transfection

COS-7 and SH-SY5Y cells were purchased from the Riken Cell Bank (Tsukuba, Japan). Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from Sumitomo Dainippon Pharma Co., Ltd. (Osaka, Japan). COS-7 cells were cultured in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan), and SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 medium (Wako, Osaka, Japan). These media for COS-7 and SH-SY5Y cells contained 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). HUVECs were cultured in EBM-2 medium (CAMBREX, East Rutherford, USA) containing Clonetics

EGM-2 BulletKit (CAMBREX). Culturing was performed in a humidified atmosphere containing 5% CO_2 at 37 °C.

For the transfection of plasmids into cultured cells, the expression plasmids were electroporated using an electroporator NEPA21 (NEPA GENE, Chiba, Japan) according to the protocol recommended by the supplier. Briefly, 10 μg plasmids were transfected into 2×10^6 cells, and transfected cells were seeded on the appropriate culture dishes.

For adenoviral vector infection, SH-SY5Y cells (5×10^5) seeded on glass-bottom culture dishes were incubated in serum-free medium for 2 h with recombinant adenovirus vectors with a multiplicity of infection of 5. After infection, the viral supernatant was removed, and the cells were cultured in normal serum-containing medium.

2.5. Observation of PKC translocation

PKC translocation was observed 2 days after transfection with PKC-GFPs or δPKC-DsRed. The culture medium of the SH-SY5Y cells or HUVECs expressing PKC-GFPs was replaced with normal HEPES buffer 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 fluorescent protein-tagged PKCs was triggered by the addition of the various stimuli to the HEPES buffer to obtain the appropriate final concentration. The GFP or DsRed fluorescence was monitored with a fluorescence microscope (BZ900, Keyence, Osaka, Japan). Time-series images were obtained after the application of propofol.

In advance to the PKC translocation studies, we preliminarily performed several experiments that determined the threshold concentration of propofol that reliably elicits the translocation of each PKC. We used this threshold concentration of propofol for later experiments.

2.6. In vitro PKC kinase assay

Two days after transfection by electroporation, COS-7 cells expressing PKC-GFPs were harvested. The cells were centrifuged and re-suspended in 400 μl of homogenization buffer (250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 20 mM Tris—HCl, 200 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4). After sonication, samples were centrifuged (20,000 × *g* for 15 min at 4 °C), and the supernatant was collected.

For the immunoprecipitation of PKC-GFPs, an equivalent amount of supernatant was rotated with the anti-GFP antibody (1 h at 4 °C) and then precipitated with protein A-Sepharose for an additional 1 h. The beads were collected and washed five times with phosphate-buffered saline (PBS). Finally, 10 μl of the suspended pellet was used for the kinase assay.

PKC activity was assayed by measuring the incorporation of ^{32}Pi from [γ^{32}P]ATP into calf thymus H1 histone in a reaction mixture containing 20 mM Tris•HCl at pH 7.5, 10 mM $MgCl_2$, 20 μM ATP, 15—50 kBq of [γ^{32}P]ATP, and 200 μg/ml H1 histone. The incubation was carried out for 5 min at 30 °C, and the phosphorylated proteins were separated by SDS/PAGE and visualized and quantified by measurement of photostimulated luminescence intensity with a Bio-Imaging Analyzer (FLA-2000, Fuji). The PKC activity was measured in the presence of DMSO (control), 0.8 μM TPA (positive control) or the desired concentration of propofol.

2.7. Statistical analysis

Statistics were obtained using Prism 4 software (GraphPad Software, San Diego, CA). Statistical significance was determined by one-way ANOVA followed by Dunnett's post-test. If the P value was less than 0.05 ($P < 0.05$), the difference was considered significant.

3. Results

3.1. Propofol-induced translocation of PKC

For the series of studies on propofol-induced PKC translocation, PKC-GFPs or δPKC-DsRed were expressed in SH-SY5Y cells, a human neuroblastoma cell line, by adenoviral vector infection. PKC translocation was triggered by the application of propofol at the appropriate concentration.

3.2. Propofol-induced translocation of conventional PKC (cPKC)

We observed the translocation of γPKC-GFP, a neuron-specific subtype of cPKC fused with GFP. As shown in Fig. 2, representative images from 5 experiments, γPKC-GFP was gradually translocated from the cytosol to the plasma membrane by 400 μM propofol within 15 min. Because propofol at concentrations less than 400 μM did not induce translocation, we determined that the threshold concentration of propofol for γPKC translocation induction was approximately 400 μM. We also found that αPKC-DsRed, another subtype of cPKC, showed propofol-induced translocation similar to γPKC-GFP (Supplemental figure 1, representative images from 4 experiments).

3.3. Propofol-induced translocation of novel PKCs (nPKCs)

We first examined the translocation of δPKC, which is ubiquitously expressed in tissues including the endothelia and brain. As shown in Fig. 3A, representative images from 4 experiments, 400 μM propofol gradually translocated δPKC-DsRed from the cytosol to the perinuclear region within 15 min. Next, we investigated the translocation of εPKC, which is expressed in the endothelia, brain and heart. The propofol-induced translocation of εPKC-GFP was very complicated (Fig. 3B, representative images from 6 experiments). After the application of 200 μM propofol, εPKC-GFP first accumulated in the perinuclear region within 1.5 min. Subsequently, εPKC-GFP was translocated to the nucleus within 3 min. Thereafter, it moved to the plasma membrane within 7 min. εPKC-GFP still remained in the nucleus but no longer accumulated in the perinuclear region 7 min after application (Supplemental video 1). Furthermore, propofol (200 μM) induced the translocation of ηPKC-GFP to the vesicle-like intracellular organelle (Supplemental figure 2, representative images from 3 experiments). The threshold concentration of propofol for the nPKCs tested in these experiments was approximately 200 μM, which was relatively smaller than the threshold for cPKCs.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.jphs.2018.03.008.

3.4. Propofol-induced translocation of atypical PKC (aPKC)

Among the aPKCs, we investigated the translocation of ζPKC, which is also ubiquitously expressed in the endothelia and brain. ζPKC-GFP in the cytosol was unidirectionally translocated to the nucleus within 4 min (Supplemental figure 3, representative images from 4 experiments). The threshold concentration of propofol for ζPKC translocation was approximately 200 μM.

Overall, the propofol-induced translocation of cPKC and aPKC was unidirectional. In contrast, the translocation of nPKCs was

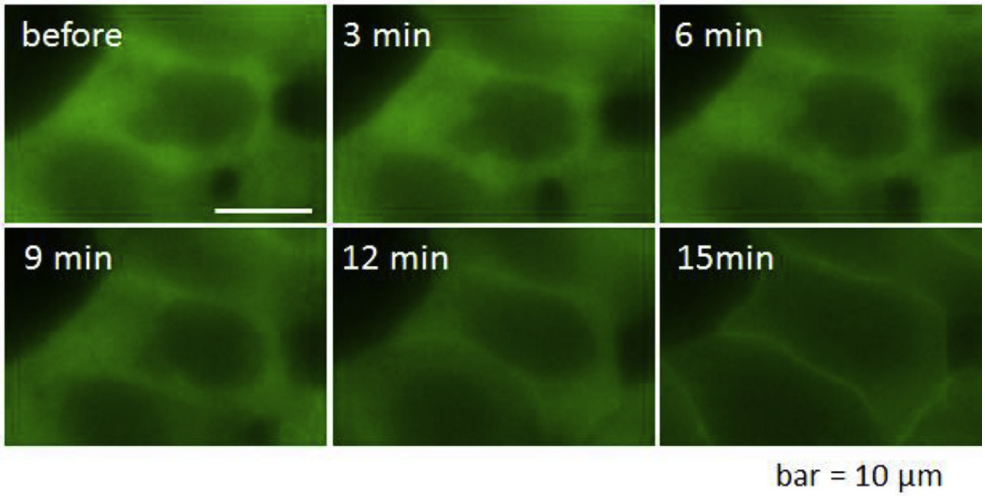

Fig. 2. Time-series observation of the propofol-induced translocation of γPKC-GFP expressed in SH-SY5Y cells. The translocation of γPKC-GFP was triggered by the application of 400 μM propofol. The figures are representative images from 5 experiments. γPKC-GFP was gradually translocated to the plasma membrane.

diverse among the subtypes, suggesting that propofol induced subtype-specific translocation of PKCs.

3.5. Role of the C1 domain in propofol-induced translocation of εPKC

For the series of studies on the role of the C1 domain in propofol-induced translocation, various types of C1 domain mutants of εPKC-GFP were expressed in SH-SY5Y cells by electroporation of plasmids.

The propofol-induced translocation of εPKC was complicated and diverse (Fig. 3B). As propofol is lipophilic, this chemical is postulated to act directly on the C1 domain, a lipid-binding domain of PKC, and regulate PKC translocation. To elucidate the role of the C1 domain in the propofol-induced translocation of εPKC, we used deletion mutants of the C1 domain in εPKC.[12] We prepared three types of mutants that lacked both or either of the C1a and C1b domains (Fig. 4). We investigated the propofol-induced translocation of each mutant εPKC-GFP.

Again, 200 μM propofol induced diverse translocation of wild-type εPKC-GFP to the perinuclear region, nucleus and plasma membrane (Fig. 5A, representative images from 6 experiments). As shown in Fig. 5B, representative images from 4 experiments, C1a and C1b-deleted εPKC (ΔC1aC1bεPKC) was unidirectionally translocated to the nucleus, not to either the perinuclear region or the plasma membrane. In contrast, C1a-deleted εPKC (ΔC1aεPKC) tended to accumulate in the perinuclear region (Fig. 5C, representative images from 4 experiments, before). The application of 200 μM propofol further induced ΔC1aεPKC-GFP accumulation in the perinuclear regions within 4 min (Fig. 5C, 4 min). Thereafter, the accumulated ΔC1aεPKC-GFP was gradually translocated to the plasma and nuclear membrane within 10 min (Fig. 5C, 10 min). Very faint intranuclear translocation was also observed (Fig. 5C, 8 and 10 min). We next investigated the propofol-induced translocation of C1b-deleted εPKC (ΔC1bεPKC) (Fig. 5D, representative images from 4 experiments). The translocation of ΔC1bεPKC-GFP was very similar to that of ΔC1aC1bεPKC-GFP; that is, cytosolic ΔC1aC1bεPKC-GFP was unidirectionally translocated to the nucleus, not to either the perinuclear region or the plasma membrane. These results suggest that the C1 domain was not required for the nuclear translocation of εPKC by propofol. In contrast, the C1b domain was considered to be indispensable for the propofol-induced translocation to the perinuclear region and plasma membrane.

3.6. Role of the C1 domain in propofol-induced translocation of γPKC

To investigate the role of the C1 domain in the propofol-induced translocation of γPKC, we used the mutant γPKC, the lipid-binding sites in the C1a and C1b domains of which were lost (non-lipid-biding γPKC).[8] As shown in Fig. 6, representative images from 3 experiments, the propofol-induced translocation of non-lipid-biding γPKC-GFP was almost similar to that of wild-type γPKC-GFP (Fig. 2), suggesting that the C1 domain was not required for the propofol-induced translocation of γPKC.

3.7. Propofol-induced translocation of εPKC in endothelial cells (HUVECs)

Propofol is considered to exert its anesthetic effects in the central nervous system, while some adverse effects are supposed to occur in peripheral tissues including endothelial cells. Therefore, we attempted to observe the propofol-induced translocation of εPKC-GFP in HUVECs, an endothelial cell line. As shown in Fig. 7, representative images from 3 experiments, after the application of 200 μM propofol, εPKC-GFP was accumulated as puncta in the perinuclear regions within 2 min. Thereafter, the margin of the nucleus became obscure, probably due to the translocation εPKC-GFP to the nucleus within 5 min. Faint translocation to the plasma membrane was seen 5 or 10 min after the application (arrow head). This translocation pattern of εPKC-GFP was very similar to the translocation seen in SH-SY 5Y cells.

3.8. Regulation of PKC kinase activity by propofol

PKC is considered to be translocated to the desired target regions by the guidance of lipid mediators such as arachidonic acid and ceramide, and there, PKC is activated and phosphorylates the target substrate. To elucidate the physiological significance of propofol-induced PKC translocation, it is necessary to know whether propofol regulates PKC activity when it was translocated

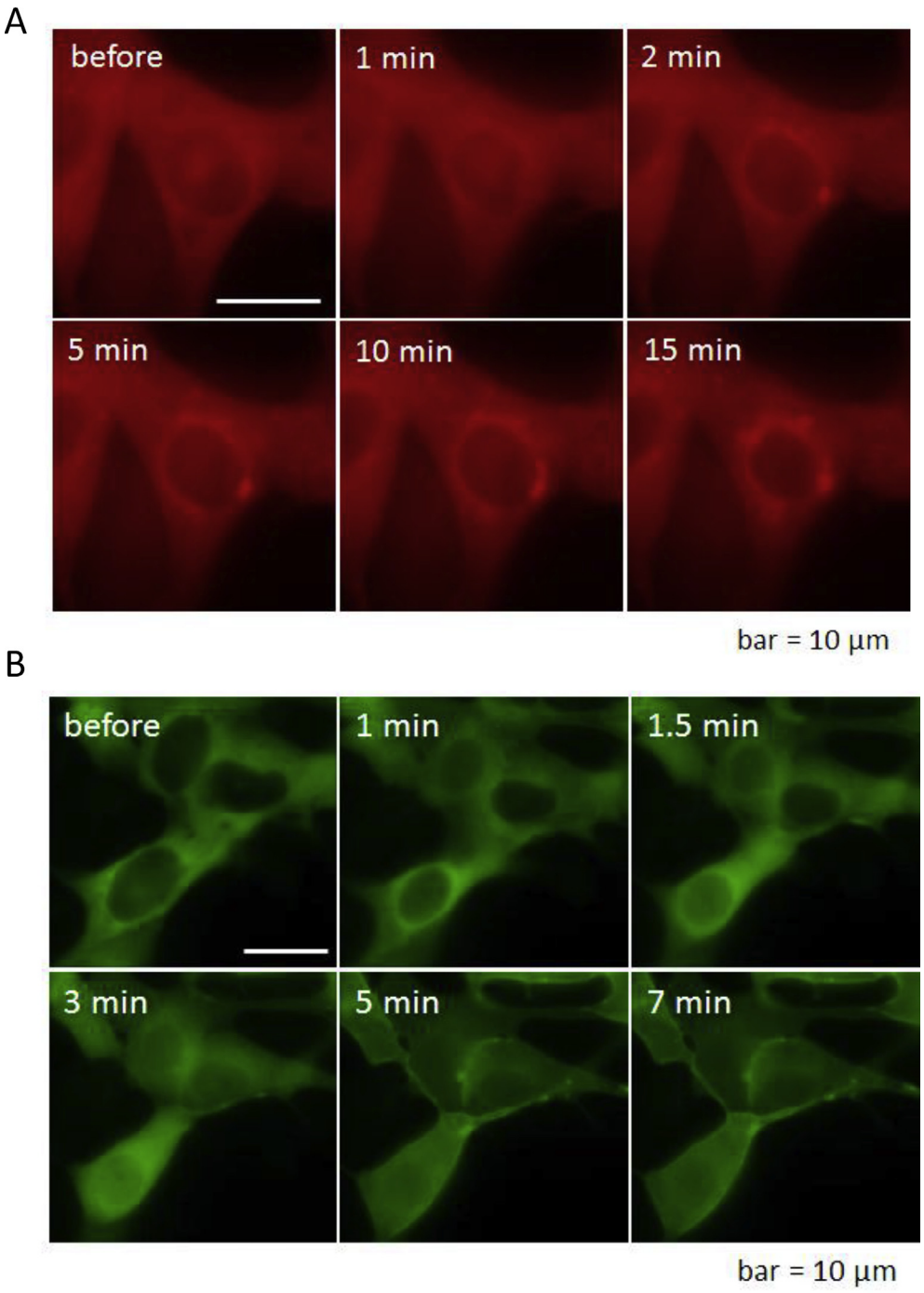

Fig. 3. A: Time-series observation of the propofol-induced translocation of δPKC-DsRed expressed in SH-SY5Y cells. The translocation of δPKC- DsRed was triggered by the application of 200 μM propofol. The figures are representative images from 4 experiments. δPKC- DsRed was gradually translocated to the perinuclear region. B: Time-series observation of the propofol-induced translocation of εPKC-PKC expressed in SH-SY5Y cells. The translocation of εPKC-GFP was triggered by the application of 200 μM propofol. The figures are representative images from 6 experiments. εPKC-GFP was first translocated to the perinuclear region (1 and 1.5 min), then to the nucleus (3 min) and finally to the plasma membrane (5 and 7 min).

to the target site. For this purpose, we performed an in vitro PKC kinase assay. We selected γPKC and εPKC as a representative of the cPKC and nPKC families, respectively. We examined the effects of 100–1000 μM propofol, which covered the threshold concentration for PKC translocation. The results revealed that 100 μM propofol significantly increased the activities of both γPKC and εPKC (Fig. 8). Propofol at the range of 200–500 μM tended to increase the activity of both γPKC and εPKC. In contrast, propofol at the concentration of 1000 μM significantly reduced the activity of γPKC and εPKC (Fig. 8).

We also investigated the in vitro PKC kinase assay for αPKC, δPKC ηPKC and ζPKC, other than γPKC and εPKC. The effects of propofol on the kinase activities of these PKC subtypes are very similar to those of γPKC and εPKC although a statistical significance was not obtained in αPKC (Supplemental figure 4).

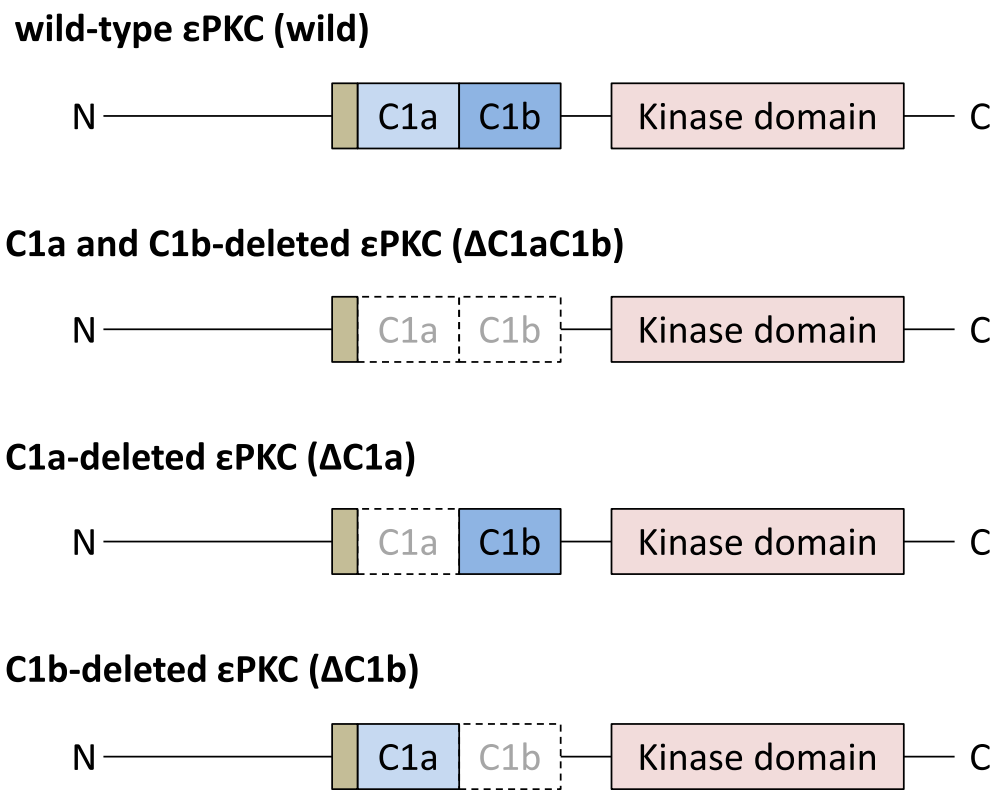

Fig. 4. Structure of the C1 domain-deleted mutant εPKC used in this study. We prepared C1a and C1b-deleted, C1a-deleted and C1b-deleted εPKC.

4. Discussion

4.1. Propofol-induced translocation of PKC families

In this study, we investigated whether propofol induced PKC translocation using cPKC, nPKC and aPKC fused with fluorescent protein. The propofol-induced translocation of cPKCs including αPKC and γPKC was unidirectional; namely, propofol translocated cPKCs from the cytosol to the plasma membrane. In contrast, the propofol-induced translocation of nPKCs, including δPKC, εPKC and ηPKC, was diverse and subtype specific. Propofol translocated δPKC to the perinuclear region8 (Fig. 3A), and it translocated ηPKC to a vesicular organelle of unknown origin (Supplemental Figure 2). The translocation of εPKC was especially complicated and diverse (Figs. 3B and 5A).

Propofol translocated δPKC and εPKC to the perinuclear region (Fig. 3A and B). Our previous study revealed that ceramide or arachidonic acid, two lipid mediators, translocated δPKC or εPKC, respectively, to the perinuclear organelle, the Golgi apparatus.[11–13] As this translocation was very similar to that induced by propofol, the perinuclear region where δPKC or εPKC was targeted was likely the Golgi apparatus. Also, these findings suggest that the lipophilic chemical propofol induces diverse PKC translocation, just as lipid mediators such as ceramide and arachidonic acid do for the nPKC family.

We examined the threshold concentration of propofol that could induce PKC translocation. The value was approximately 400 μM for cPKC and approximately 200 μM for nPKC and aPKC, suggesting that nPKC and aPKC have a relatively higher sensitivity for propofol-induced translocation.

4.2. Roles of the C1 domain in the propofol-induced translocation of PKCs

The propofol-induced translocation of εPKC was very complicated. The time-series observation demonstrated that εPKC was first moved to the perinuclear region, then to the nucleus, and finally to the plasma membrane. To elucidate whether the C1 domain, a lipid-binding domain, was involved in this complicated translocation of εPKC, we tested various types of mutant εPKC whose C1a or C1b domain was deleted (Fig. 4). Judging from the results as shown in Fig. 5, the C1b domain was indispensable for the propofol-induced εPKC translocation to the perinuclear region and plasma membrane. Notably, the C1b domain of nPKC has already been identified as important for lipid mediator-induced translocation since this domain had an important role in its translocation induced by ceramide and arachidonic acid.[11–13] The sensitivity of the C1b domain to propofol might determine the diversity and subtype specificity of the propofol-induced translocation of nPKCs.

In contrast, εPKC translocation to the nucleus did not depend on the C1 domain. The nuclear localization signal has generally been considered to play an important role in nuclear protein translocation. Although whether εPKC has a functional nuclear localization signal remains unclear, propofol may directly or indirectly regulate this signal, thereby affecting εPKC translocation to the nucleus. ζPKC was also translocated to the nucleus by propofol (Supplemental figure 3). Further analysis is necessary to clarify the molecular mechanism underlying the nuclear translocation of εPKC and ζPKC induced by propofol.

We also investigated the role of the C1 domain in the propofol-induced translocation of γPKC, a member of the cPKC family. We

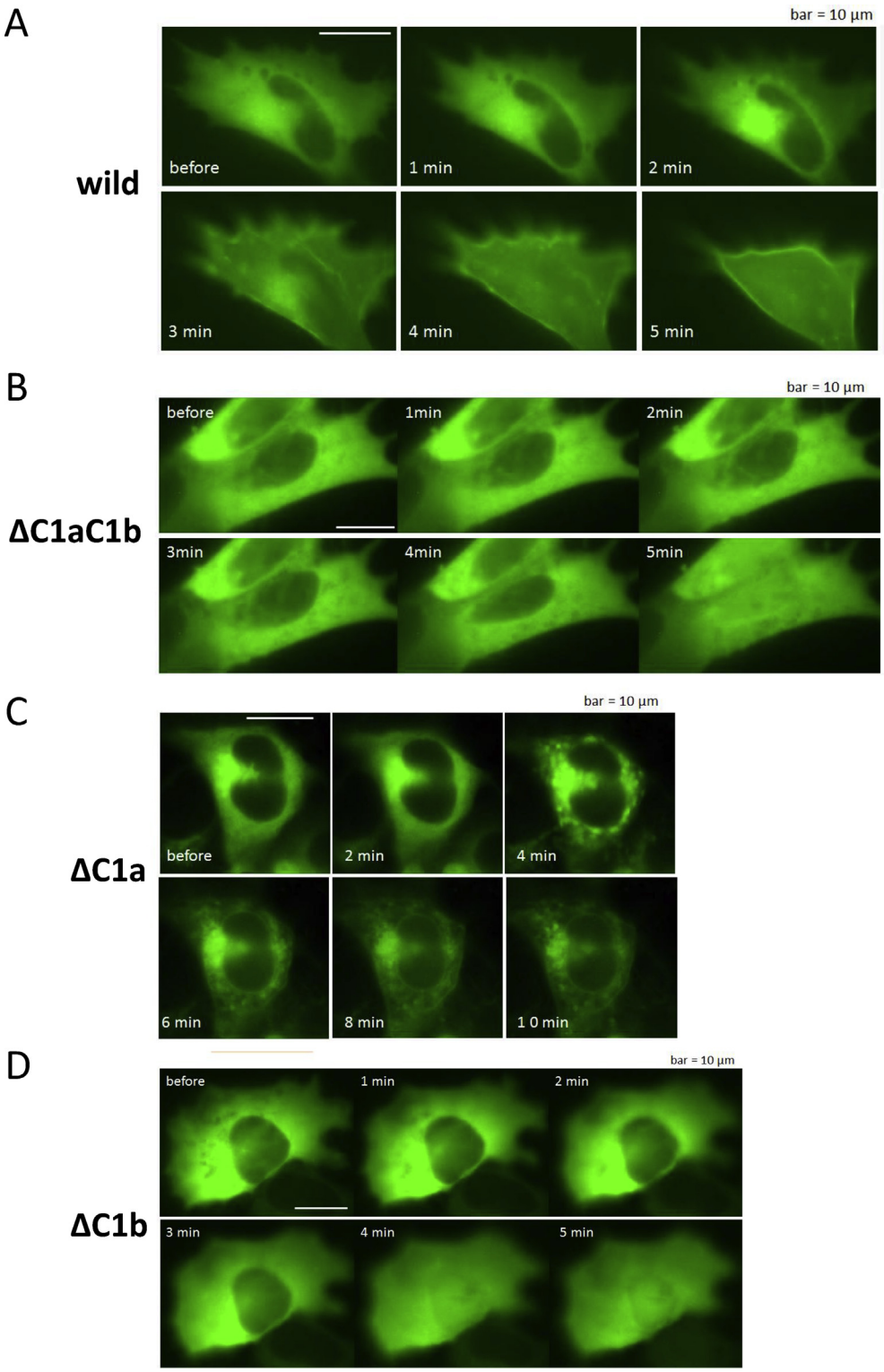

Fig. 5. A: Time-series observation of the propofol-induced translocation of wild-type εPKC-PKC expressed in SH-SY5Y cells. The translocation of εPKC-GFP was triggered by the application of 200 μM propofol. The figures are representative images from 6 experiments. The translocation pattern of εPKC-PKC was almost the same as that seen in Fig. 3B. B: Time-series observation of the propofol-induced translocation of ΔC1aC1b εPKC-PKC expressed in SH-SY5Y cells. The translocation of ΔC1aC1b εPKC-PKC was triggered by the application of 200 μM propofol. The figures are representative images from 4 experiments. ΔC1aC1b εPKC-PKC was translocated to the nucleus. C: Time-series observation of the propofol-induced translocation of ΔC1a εPKC-PKC expressed in SH-SY5Y cells. The translocation of ΔC1a εPKC-PKC was triggered by the application of 200 μM propofol. The figures are representative images from 4 experiments. ΔC1a εPKC-PKC was first translocated to the perinuclear region and thereafter to the plasma and nuclear membrane. D: Time-series observation of the propofol-induced translocation of ΔC1b εPKC-PKC expressed in SH-SY5Y cells. The translocation of ΔC1b εPKC-PKC was triggered by the application of 200 μM propofol. The figures are representative images from 4 experiments. ΔC1b εPKC-PKC was translocated to the nucleus.

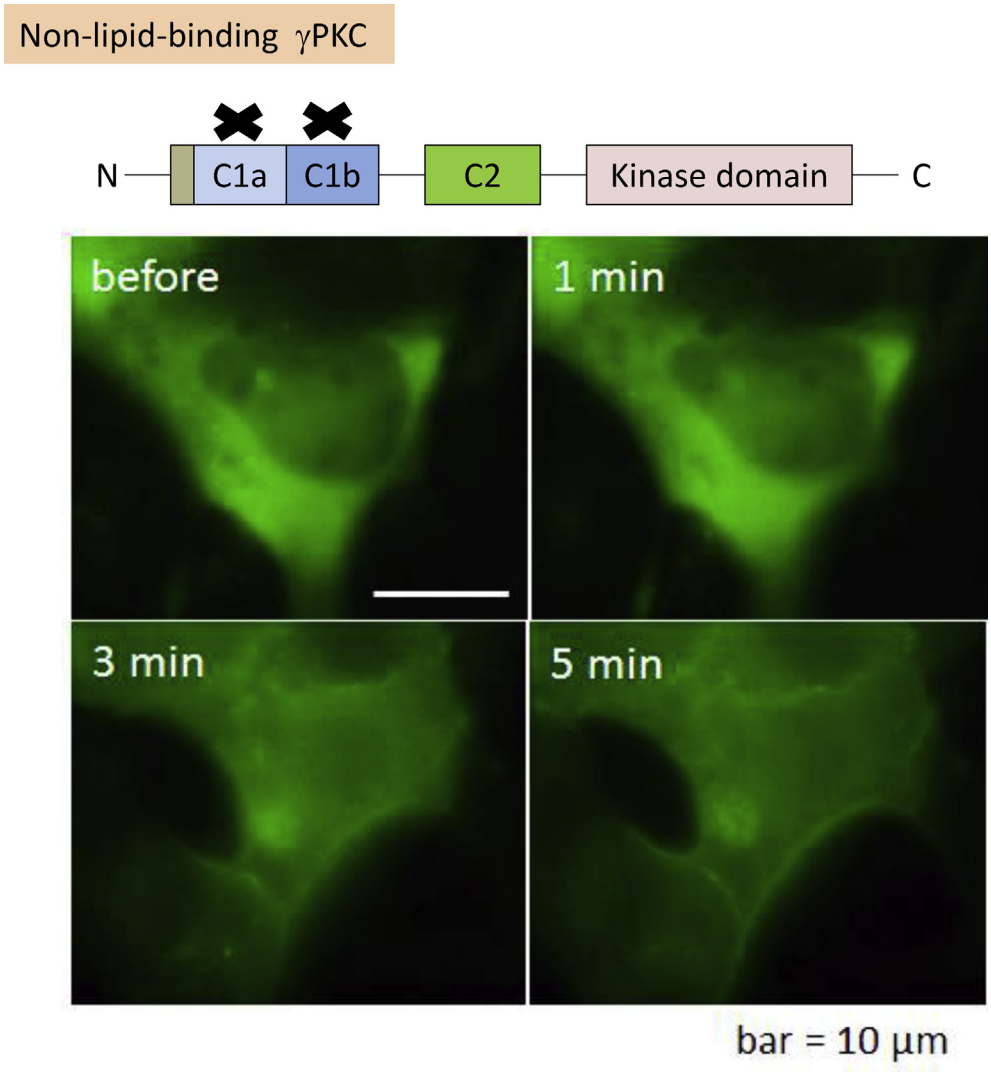

Fig. 6. Time-series observation of the propofol-induced translocation of lipid-binding less γPKC-PKC expressed in SH-SY5Y cells. The translocation of non-lipid-biding γPKC-PKC was triggered by the application of 400 μM propofol. The figures are representative images from 3 experiments. Non-lipid-biding γPKC-PKC was translocated to the plasma membrane.

also used a mutant γPKC that lacked the ability to bind to lipids.[8] Interestingly, this mutant could be translocated to the plasma membrane by propofol, suggesting that the C1 domain, a lipid-binding domain, was not involved in the propofol-induced translocation of γPKC. Our preliminary study revealed that propofol at 100 μM immobilized the intracellular calcium; therefore, the C2 domain, a calcium-binding domain, may play an important role in the propofol-induced translocation of γPKC.

Further experiments using other types of PKC are necessary to confirm the role of C1 domain in the propofol-induced translocation of each subtype of PKC.

4.3. Regulation of PKC activity by propofol

PKCs are translocated to target sites by a variety of stimulants, and there, they are activated, followed by their phosphorylation of substrates. Therefore, PKCs must be activated at target sites to exert their physiological functions. To elucidate whether propofol could regulate the kinase activity of PKCs, we carried out an in vitro kinase assay. The results demonstrated that γPKC and εPKC, tested in this study, are activated or tented to be activated around the threshold concentration for their translocation, suggesting that propofol could translocate PKCs to their appropriate target sites and concomitantly activate PKCs at these sites. Considering the difference in dose dependency of propofol-induced PKC activation in vitro and in vivo, further investigation such as in vivo kinase assay would be necessary to confirm whether PKCs are activated at the target sites by propofol.

4.4. Physiological significance of the propofol-induced translocation of PKCs

Propofol-induced anesthesia is generally accepted to occur at a blood concentration of 4–6 mg/l, which corresponds to 22–34 μM.[21] This fact suggests that the clinical application of propofol does not reach the threshold concentration for PKC translocation, near 200 μM, in the tissue, including the brain. Nonetheless, the vessels and endothelium near the region where the propofol is applied may be exposed to a high concentration of propofol near the threshold for PKC translation.

The pain associated with a propofol injection is the most common adverse effects of propofol.[22] It is known that the contact of

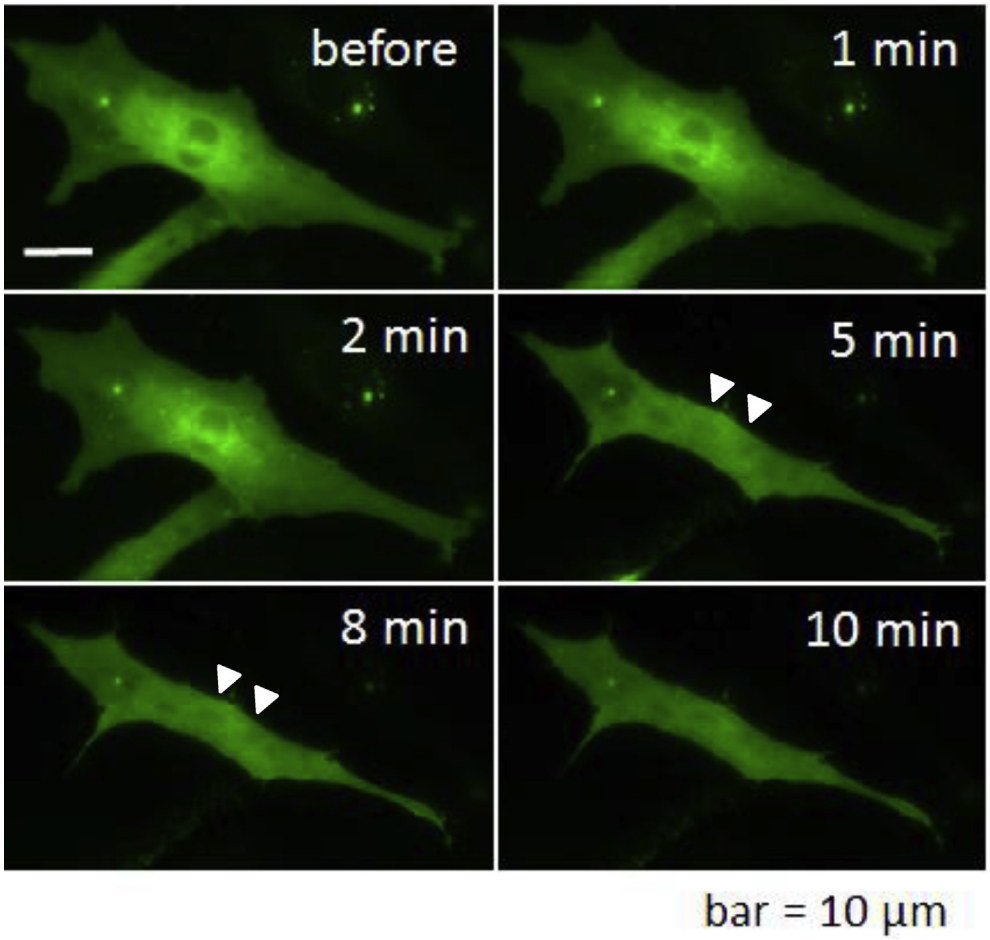

Fig. 7. Time-series observation of the propofol-induced translocation of εPKC-PKC expressed in HUVECs. εPKC-GFP was expressed in HUVECs by plasmid electroporation. The translocation of εPKC-GFP was triggered by the application of 200 μM propofol. The figures are representative images from 3 experiments. εPKC-GFP was first accumulated in the perinuclear region (1 and 1.5 min) and was then translocated to the nucleus (5 min). Faint translocation of εPKC-GFP to the plasma membrane was seen (arrow head, 5 and 8 min).

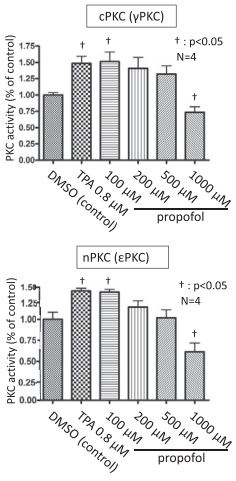

Fig. 8. In vitro kinase activity of γPKC and εPKC in the presence or absence of propofol. Each column represents the PKC activity as the percent of activity in the DMSO-treated control. The effect of TPA (0.8 μM) on the PKC activity was tested as a positive control. Propofol at 100 μM; significantly increased the kinase activity of both γPKC and εPKC ($N = 4$. † $P < 0.05$, one-way ANOVA followed by Dunnett's post-test). In addition, 200 and 500 μM; propofol tended to increase the kinase activity of both γPKC and εPKC.

propofol with the vein wall is important for the occurrence of pain on propofol injection.[23] In addition, various TRP channels, important molecules for pain signaling, are well known substrates of PKCs, which positively regulates the TRP channels functions.[24] In fact, propofol at a concentration of 500 μM has been reported to elicit TRPA1-mediated pain behaviors in mice.[25] These findings lead the possibility that the propofol-induced PKCs translocation is involved in the cause of pain on propofol injection.

Therefore, we investigated PKC translocation in HUVECs, an endothelial cell line in order to consider the physiological and clinical significance of propofol-induced PKCs translocation. We confirmed that εPKC was also translocated in HUVECs, indicating the possible involvement of PKC translocation in the exertion of the beneficial or adverse effects induced by propofol in peripheral tissues, especially the vein. Because propofol is a lipophilic chemical and can easily pass through the blood–brain barrier, accumulated propofol in the brain could induce the translocation of PKCs in cells of the central nervous system.

In addition to the $GABA_A$ receptor, propofol is known to interact with the glycine receptor,[26] serotonin type 3 receptor[27] and glutamate receptors,[28] exerting its effects via modulating these receptors. Since these receptors are reported to be modulated by PKC activation,[26–28] the propofol-induced PKC translocation might be involved in the emergence of beneficial or adverse effects of propofol via these receptors.

In conclusion, we demonstrated that propofol induced diverse and subtype-specific translocation of PKCs. The propofol-induced translocation of PKCs may contribute to the exertion of the beneficial or adverse effects mediated by propofol on a variety of organs.

Conflict of interest

Authors have no conflict of interest regarding this study.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jphs.2018.03.008.

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