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Identification of protein kinase C domains involved in its translocation induced by propofol

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Abstract

Propofol is widely used for general anesthesia and sedation; however, the mechanisms of its anesthetic and adverse effects are not fully understood. We have previously shown that propofol activates protein kinase C (PKC) and induces its translocation in a subtype-specific manner. The purpose of this study was to identify the PKC domains involved in propofol-induced PKC translocation. The regulatory domains of PKC consist of C1 and C2 domains, and the C1 domain is subdivided into the C1A and C1B subdomains. Mutant PKC α and PKC δ with each domain deleted were fused with green fluorescent protein (GFP) and expressed in HeLa cells. Propofol-induced PKC translocation was observed by time-lapse imaging using a fluorescence microscope. The results showed that persistent propofol-induced PKC translocation to the plasma membrane was abolished by the deletion of both C1 and C2 domains in PKC α and by the deletion of the C1B domain in PKC δ. Therefore, propofol-induced PKC translocation involves the C1 and C2 domains of PKC α and the C1B domain of PKC δ. We also found that treatment with calphostin C, a C1 domain inhibitor, abolished propofol-induced PKC δ translocation. In addition, calphostin C inhibited the propofol-induced phosphorylation of endothelial nitric oxide synthase (eNOS). These results suggest that it may be possible to modulate the exertion of propofol effects by regulating the PKC domains involved in propofol-induced PKC translocation.

Keywords

Propofol, protein kinase C, translocation, endothelial nitric oxide synthase (eNOS)

1. Introduction

Propofol is an intravenous anesthetic frequently used for general anesthesia and sedation. Its anesthetic effect is thought to be mainly mediated via activation of gamma-aminobutyric acid A ($GABA_A$) receptors (Franks, 2006).

Propofol is a useful anesthetic because it is capable of rapid induction and arousal (Sahinovic et al., 2018), but it has several adverse effects. The most common are vascular pain during injection and hypotension (Desousa, 2016), possibly resulting from excessive vasodilation. The less common but more serious is propofol infusion syndrome (PRIS) (Hemphill et al., 2019), the causes of which are not fully understood.

Protein kinase C (PKC) is a family of serine-threonine kinases that are involved in various intracellular signal transduction pathways. There are more than 10 PKC isoforms, which are classified into three subgroups based on their structures: conventional PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC)(Tanaka and Nishizuka, 1994). PKC consists of two common domains, the kinase domain and the regulatory domain, and its activity is controlled by the regulatory domain.

The regulatory domains include C1 and C2 domains. The C1 domain is a lipid-binding domain subdivided into C1A and C1B; it activates PKC by binding to lipids (e.g., diacylglycerol) or PKC activators (e.g., phorbol esters). The C2 domain is a Ca^{2+}-binding domain that activates PKC by binding to the plasma membrane in a Ca^{2+}-dependent manner (Newton and Johnson, 1998).

Because cPKC has both C1 and C2 domains, its activity is regulated by both lipids and Ca^{2+}. As nPKC has a C1 domain, not a Ca^{2+}-binding C2 domain, its activity is regulated solely by lipids (Lipp and Reither, 2011).

Upon various stimulations, including receptor activation and lipid mediators, PKC localization is altered and PKC is targeted to specific subcellular regions. Thereafter, PKCs phosphorylate the substrates at their target sites. This phenomenon is known as PKC translocation (Shirai and Saito, 2002). In cPKC, stimulation of the G protein-coupled receptor activates phospholipase C, which degrades phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate, mobilizing Ca^{2+} from the ER. Diacylglycerol and Ca^{2+} bind to the C1 and C2 domains, respectively, and translocate PKC to the plasma membrane (Igumenova, 2015).

PKC is also translocated by various lipids, the features of which depend on the lipid and PKC isoform (Kajimoto et al., 2001; Kashiwagi et al., 2002; Shirai et al., 1998). Such lipid- and isoform-specific PKC translocation is important for understanding the mechanism of the spatiotemporal regulation of PKC (Shirai and Saito, 2002).

In a previous study, we showed that propofol activated PKC in vitro and induced isoform-

specific translocation of PKCs (Miyahara et al., 2018). Propofol has also been shown to release Ca^{2+} from the ER and increase intracellular Ca^{2+} concentration (Urabe et al., 2020), which may contribute to C2 domain activation in cPKC. Thus, activation of PKC and induction of PKC translocation by propofol may be involved in the anesthetic's action. However, it is unclear which PKC domains are involved. Therefore, the purpose of this study was to identify the domains involved in propofol-induced PKC translocation and to obtain a better understanding of the effects of propofol on PKC.

2. Materials and methods

2.1. Materials

Propofol was obtained from FUJI FILM Wako Pure Chemical (Osaka, Japan). Calphostin C was purchased from EMD Millipore (Burlington, MA, USA). Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Bryostatin1 was obtained from Merck (Darmstadt, Germany). Anti-eNOS (endothelial nitric oxide synthase) rabbit polyclonal antibody was purchased from GeneTex (Irvine, CA, USA). Anti-phospho-eNOS (peNOS) rabbit polyclonal antibody was purchased from Cell Signaling Technology (Danvers, MA, USA), and glass-bottom culture dishes were purchased from MatTek Corporation (Ashland, OR, USA). All other chemicals used were of analytical grade.

2.2. Cell culture

HeLa cells were purchased from the Riken Cell Bank (Tsukuba, Japan). HUVECs were purchased from ATCC (Manassas, VA, USA). Endothelial cell growth supplement (ECGS) was purchased from Sigma-Aldrich (Tokyo, Japan). HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; FUJIFILM Wako, Osaka, Japan) supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). HUVECs were cultured in DMEM/F12 supplemented with 10% fetal calf serum (FCS), penicillin (100 units/mL), streptomycin (100 μg/mL), ECGS (40 μg/mL), and heparin (1mg/mL). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 ° C in the dark.

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

2.3. Construction of PKC domain deletion mutants

Plasmids encoding PKCα-GFP (green fluorescent protein) and PKCδ-GFP were generated as previously described (Sakai et al., 1997) (Ohmori et al., 1998). Domain-deleted mutants were created using a KOD-Plus-Mutagenesis Kit (TOYOBO, Osaka, Japan). Briefly, inverse PCR was performed using the plasmid DNA of PKCα-GFP or PKCδ-GFP as a template with primers set outside the domain to be deleted. After digestion of the template plasmid with the restriction enzyme DpnI, the PCR product was self-ligated. The plasmid was

then transformed into competent DH5α cells and amplified.

2.4. Observation of propofol-induced PKC translocation

Propofol-induced PKC translocation was observed as previously described (Miyahara et al., 2018). Two days after transfection, the culture medium was replaced with normal HEPES buffer composed of 165 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, and 10 mM glucose (pH 7.4). To observe propofol-induced PKC translocation, time-lapse imaging was performed using a fluorescence microscope (KETENCE, BZ-9000, Osaka, Japan) after propofol application at a final concentration of 100 μM. The observations were performed at room temperature (20~25℃) . Owing to the high lipid solubility of propofol at room temperature, sonication was applied to the propofol/DMSO-diluted solution immediately before application.

PKC translocations induced by PMA (1 μM) or bryostatin1(500 nM), PKC activators, were also observed for comparison with propofol-induced PKC translocation.

To observe the effect of calphostin C on propofol-induced PKC translocation, cells were pre-treated with calphostin C (2 μM) or vehicle (DMSO) for 15 min at 37° C before propofol application.

2.5. Western blotting

Western blotting was performed as previously described (Taguchi et al., 2021). Briefly, cell lysate samples were electrophoretically separated by 9% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Burlington, MA, USA). The PVDF membranes were then incubated with an anti-eNOS rabbit polyclonal antibody (diluted 1:1000) or anti-peNOS rabbit polyclonal antibody (diluted 1:1000) for 16 h at 4° C. After three washes with PBS-T (0.01 M phosphate-buffered saline containing 0.03% Triton X-100), the membranes were incubated with an HRP-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA, diluted 1:10,000) for >1 h at room temperature. Immunoreactive bands were visualized using a chemiluminescence detection kit (Chemi-Lumi One, Nacalai Tesque, Kyoto, Japan). The band densities were measured using a luminescent image analyzer (EZ-Capture MG, ATTO, Tokyo, Japan). The expression levels of peNOS and eNOS were quantified using a CS Analyzer (ATTO, Tokyo, Japan), and the peNOS/eNOS ratio was considered the phosphorylation ratio of eNOS.

2.6. Statistical analysis

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2 Prism 4 software (GraphPad Software, San Diego, CA, USA) was used for the statistical
3 analysis. Statistical significance was determined using one-way ANOVA, followed by
4 Dunnett's post-test or unpaired t-test. Differences were considered significant when $p < 0.05$.

3. Results

To identify the PKC domains involved in propofol-induced PKC translocation, GFP was fused to mutant PKCs, each domain of which was deleted, and the PKC-GFPs were expressed in HeLa cells. In this study, we examined domain-deleted mutants of PKC α and PKC δ, which belong to the cPKCs and nPKCs groups, respectively.

3.1. Identification of domains essential for propofol-induced PKC α translocation

3.1.1. Structure and confirmation of domain-deleted mutants of PKC α

We transfected plasmids expressing various PKC α mutants, whose domains were deleted, into HeLa cells by electroporation (Fig. 1A). Western blotting analysis revealed that each PKC α mutant showed an appropriate molecular weight that was predicted by the respective deletion of the domain (Fig. S9A).

3.1.2. Translocation of wild-type (WT) and C1 domain-deleted mutants of PKC α

Two days after transfection, propofol-induced PKC α translocation was observed by fluorescence microscopy. After the application of 100 μM propofol, WT PKC α rapidly and continuously translocated to the plasma membrane (Fig. 2A). In contrast, only transient translocation was observed in the C1AB domain-deleted PKC α mutant (C1ABdeletion). PKC α first translocated to the plasma membrane and then returned to the cytoplasm (Fig. 2B). This phenomenon was also observed in the C1A domain-deleted (C1Adeletion) and C1B domain-deleted mutants of PKC α (C1Bdeletion) (Fig. S1A and S1B, respectively).

3.1.3. Translocation of C2 domain-deleted mutant of PKC α

In the C2 domain-deleted mutant, PKC α (C2deletion) was translocated to the plasma membrane and to the nucleus (Fig. 2C). Nuclear translocation was observed in almost half of the examined cells (Fig. 3C).

3.1.4. Translocation of C1 and C2 domain-deleted mutants of PKC α

In the C1AB and C2 domain-deleted mutant (C1ABC2deletion), PKC α was not translocated to the plasma membrane, but to the nucleus, in all cells examined (Fig. 2D, Fig.

3B and 3C). In the C1A and C2 domain-deleted (C1AC2deletion) (Fig. S1C) and C1B and C2 domain-deleted (C1BC2deletion) (Fig. S1D) mutants, PKC α rarely translocated to the plasma membrane (Fig. 3B). In these mutants, PKC α was also translocated to the nucleus approximately 30-40% of the cells examined (Fig. 3C).

3.1.5. Character of translocation of PKC α deletion mutants to the plasma membrane

Our results showed that the duration of propofol-induced PKC α translocation to the plasma membrane differed depending on the deleted domain. To identify the domains involved in propofol-induced PKC α translocation, we classified the duration of propofol-induced PKC translocation to the plasma membrane into three categories (Fig. 3A). PKC translocation to the plasma membrane was defined as transient when most PKC returned to the cytoplasm within 10 min after application; persistent when most protein remained at the plasma membrane within 10 min; and intermediate when PKC α remained at the plasma membrane and returned to the cytoplasm to some extent (Fig. 3A).

The C1 domain-deleted mutants more frequently showed transient translocation compared to the WT, whereas the C1 and C2 domain-deleted mutants rarely translocated to the plasma membrane (Fig. 3B). These results suggest that both C1 and C2 domains are involved in propofol-induced PKC α translocation and that the C1 domain is more involved in the persistence of PKC α translocation to the plasma membrane than the C2 domain.

3.1.6. Character of translocation of PKC α deletion mutants to the nucleus.

We classified PKC α translocation into two categories based on whether it was translocated to the nucleus (Fig. 3C). C1AB and C2 domain-deleted mutants (C1ABC2deletion) showed translocation to the nucleus in all cells examined. These results suggest that translocation to the nucleus occurred as a result of C1 and C2 domain deletion.

When C1A or C1B domains remained (C2deletion, C1AC2deletion, C1BC2deletion), translocation to the nucleus occurred in about half of the cells, whereas when the C2 domain remained, translocation to the nucleus rarely occurred (C1Adeletion, C1Bdeletion, C1ABdeletion) (Fig. 3C), suggesting that it was more likely to occur when the C2 domain was deleted.

3.1.7. Propofol-induced PKC α translocation in HUVECs

The same experiment described above was also performed using HUVECs. The results are

similar to those obtained in HeLa cells (Fig. S2).

3.1.8. PKCα translocation induced by PKC activators, PMA and bryostatin 1

To compare PKCα translocation induced by propofol with that induced by well-known PKC activators, the effects of PMA (1 μM) and bryostatin1 (500 nM) on the translocation of PKCα and its mutants were examined in HeLa calls (Fig. S3 and Fig. S4). In WT PKCα, PMA or bryostatin1 translocated PKCα-GFP to the plasma membrane more slowly than propofol (Fig. S3A and Fig. S4A, respectively).

PMA-induced PKCα translocation was observed in mutants deleted in either the C1A or C1B domains (C1Adeletion, C1Bdeletion; Fig. S3B and S3C). On the other hand, bryostatin1-induced PKCα translocation was observed in the C1B domain-deleted mutant (C1Bdeletion; Fig. S4C), but not in the C1A domain-deleted mutant (C1Adeletion; Fig. S4B). Neither PMA or bryostatin1 induced translocation of mutants with only the C2 domain (C1ABdeletion; Fig. S3D and Fig. S4D, respectively). These results suggest that the domains involved in PKCα translocation induced by PMA are C1A and C1B, while the domain involved in PKCα translocation induced by bryostatin1 is C1A.

3.2. Identification of domains essential for propofol-induced PKCδ translocation

3.2.1. Structure and confirmation of domain-deleted mutants of PKCδ

We transfected the plasmids expressing various mutants of PKCδ whose domains were deleted into HeLa cells by electroporation (Fig. 1B). Western blotting analysis revealed that each mutant PKCδ had an appropriate molecular weight that was predicted by the respective deletion of the domain (Fig S9B).

3.2.2. Translocation of WT and C1 domain-deleted mutants of PKCδ

Two days after transfection, propofol-induced PKCδ translocation was observed by fluorescence microscopy. In WT, PKCδ translocated to the Golgi apparatus, followed by translocation to the plasma membrane (Fig. 4A). In the C1A domain-deleted mutant (C1Adeletion), PKCδ was abundantly expressed in the Golgi apparatus and accumulated in the cytoplasm before propofol application. PKCδ was translocated to the plasma membrane by propofol application (Fig. S5A), similar to that seen in WT (Fig. 4A). However, propofol-induced PKC translocation was not observed in the C1B domain-deleted mutant

(C1Bdeletion) (Fig. 4B) or in the C1AB domain-deleted mutant (C1ABdeletion) (Fig. S5B). We classified PKC δ translocation into two categories based on whether it was translocated to the plasma membrane (Fig. 4C). PKC δ was not translocated in the C1B domain-deleted mutant (C1Bdeletion) (Fig. 4C), suggesting that the C1B domain is essential for propofol-induced PKC δ translocation.

3.2.3. Propofol-induced PKC δ translocation in HUVECs

The experiment described above was performed using HUVECs. These results are similar to those obtained in HeLa cells (Fig. S6).

3.2.4. PMA- and bryostatin1-induced PKC δ translocation

To compare PKC δ translocation induced by propofol with that induced by PKC activators, the effects of PMA (1 μM) and bryostatin1 (500 nM) on the translocation of PKC δ and its mutants were examined in HeLa calls (Fig. S7 and Fig. S8). In WT PKC δ, PMA translocated PKC δ-GFP to the plasma membrane (Fig. S7A). Bryostatin1 mainly translocated PKC δ-GFP to nuclear membrane(Fig. S8A).

Neither PMA nor bryostatin1 translocated PKC δ to the Golgi apparatus, which was seen in propofol-induced PKC δ translocation (Fig. 4A). In the C1B domain-deleted mutant (C1Bdeletion and C1ABdeletion), PKC δ was not translocated in either PMA or bryostatin1-treated cells (Fig. S7C and 7D, Fig. 8C and 8D). These results suggest that the essential domain involved in PMA- and bryostatin1-induced PKC δ translocation is C1B.

3.3. Inhibition of propofol-induced PKC δ translocation by calphostin C

Next, we examined the effects of calphostin C, a PKC inhibitor, on propofol-induced PKC δ translocation. Although most PKC inhibitors act by binding to the ATP-binding site of the catalytic domain, calphostin C inhibits PKC activity by binding to its C1 domain (Kobayashi et al., 1989). We predicted that calphostin C would inhibit propofol-induced PKC δ translocation by blocking the binding of propofol to the C1B domain of PKC δ. After incubation with 2 μM calphostin C for 15 min at 37° C, propofol-induced PKC δ translocation was detected. As expected, pretreatment with calphostin C inhibited propofol-induced PKC δ translocation (Fig. 5A).

3.4. Effect of calphostin C on propofol-induced phosphorylation of eNOS

eNOS in the Golgi apparatus of vascular endothelial cells produces nitric oxide (NO), which may be related to adverse effects of propofol, such as hypotension and vascular pain. Excessive vasodilation by NO is thought to be one of the causes of vascular pain at propofol injection sites. Propofol has been reported to phosphorylate eNOS (Wang et al., 2010). Because propofol translocates PKCδ to the Golgi apparatus, in which eNOS is localized, and calphostin C inhibits the translocation of PKCδ, we investigated whether eNOS phosphorylation is affected by calphostin C.

HUVECs were treated with vehicle, propofol (100 μM), propofol plus calphostin C (2 μM), or calphostin C alone for 5 min at room temperature. Treated cells were collected and subjected to western blotting using anti-eNOS or anti-peNOS antibodies. Treatment with propofol significantly enhanced the phosphorylation ratio of eNOS (peNOS/eNOS) compared to vehicle treatment, whereas significant enhancement was not observed in cells treated with calphostin C concurrently with propofol (Fig. 5B and 5C). This result suggests that the propofol-induced phosphorylation of eNOS was inhibited by calphostin C.

4. Discussion

4.1.1. Regulatory domains involved in propofol-induced PKC α translocation

The mechanism of PKC activation and translocation has not been fully elucidated; however, it depends on the PKC isoform and its activator. In cPKC, translocation is thought to be triggered by Ca^{2+}-dependent binding of the C2 domain to the plasma membrane, followed by binding of the C1 domain to the plasma membrane via membrane lipids, including diacylglycerols (Nalefski and Newton, 2001; Oancea and Meyer, 1998). The purpose of this study was to identify the regulatory domains involved in propofol-induced PKC translocation.

We first performed experiments with PKC α, a member of cPKC that has C1A and C1B domains as lipid-binding domains and the C2 domain as a Ca^{2+}-binding domain. We observed how propofol-induced persistent translocation of PKC α (Fig. 2A) was altered by the deletion of each domain.

4.1.2. Role of the C1 and C2 domains of PKC α

PKC α mutants retaining the C1 domain alone (C2 deletion) were mostly translocated to the plasma membrane (Fig. 2C and Fig. 3B), indicating that the C1 domain is involved in propofol-induced PKC α translocation to the plasma membrane.

We previously found that propofol induces Ca^{2+} release from the ER, increasing intracellular Ca^{2+} concentration (Urabe et al., 2020). In this study, we revealed that propofol can translocate a PKC α mutant expressing only the C2 regulatory domain (C1AB deletion) to the plasma membrane (Fig. 2B), indicating that the C2 domain and propofol-induced increase in intracellular Ca^{2+} are involved in this translocation.

We then focused on the duration of translocation. WT PKC α consistently translocated in more than 80% of cells (Fig. 3B). In contrast, the percentage of persistent translocation of C1 or C2 domain deletion mutants was drastically reduced, and the percentage of transient translocation was increased, especially for C1 domain deletion mutants. (Fig. 3B).

These results suggest that all domains are involved in persistent translocation, and that deletion of each domain impairs PKC affinity for the membrane, reducing the duration of translocation. Deletion of either the C1A or C1B domains greatly increases the rate of transient translocation, indicating that these domains contribute most strongly to persistence of translocation.

Experiments with PKC γ-GFP by Oancea and Meyer showed that rapid PKC binding to plasma membrane requires an increase in intracellular Ca^{2+}, and subsequent stable binding

requires diacylglycerol or phorbol ester, consistent with our results (Oancea and Meyer, 1998).

What factors act on the C1 domain to induce translocation? Propofol may lead to the production of diacylglycerol or propofol itself may bind to the C1domain at the membrane. In our previous report, propofol significantly increased the activity of PKC γ and PKC ε in vitro (Miyahara et al., 2018). It has also been recently reported that administered propofol accumulates in the plasma membrane (Oda et al., 2022). Considering these findings, propofol itself may induce PKC translocation by binding to the C1 domain.

4.1.3. Role of C1A and C1B domains of PKC α

Translocation did not occur in PKC α with either the C1A or C1B domain alone as the regulatory domain (C1BC2 deletion, C1AC2 deletion) (Fig. S1C and S1D, Fig. 3B). Furthermore, deletion of either or both C1A and C1B domains shortened the duration of translocation compared to WT, but there was no significant difference in the duration of translocation between the respective mutants (C1Adeletion, C1Bdeletion, and C1ABdeletion) (Fig. 3B).

It has been reported that in PKC α, the C1A domain, has a high affinity for diacylglycerol and the C1B domain for phorbol ester (Ananthanarayanan et al., 2003). Because neither the C1A nor the C1B domain seemed to play a dominant role for propofol in the present study, we hypothesized that both domains are involved in propofol-induced PKC translocation, suggesting that one domain alone is insufficient for membrane binding and that both domains are required for persistent translocation.

In summary, propofol-induced PKC α translocation involves all C1A, C1B, and C2 domains, and this translocation may be triggered by propofol-induced increase in intracellular Ca^{2+} and subsequent binding of Ca^{2+} to the C2 domain.

4.2. Regulatory domains involved in propofol-induced PKC δ translocation

Next, we performed experiments with PKC δ, an nPKC with C1A and C1B domains as lipid-binding domains. PKC δ does not have a Ca^{2+}-binding C2 domain and is unaffected by intracellular Ca^{2+}.

PKC δ was translocated to the Golgi apparatus and then persistently translocated to the plasma membrane by propofol (Fig. 4A). Therefore, we observed how this translocation was altered by the deletion of each domain. The results showed that deletion of the C1B domain abolished these translocations (C1Bdeletion, C1AB deletion) (Fig. 4B and Fig. S5B), indicating that this domain is essential for propofol-induced PKC δ translocation to the Golgi

apparatus and plasma membrane.

Stahelin et al. found that the C1A domain has a high affinity for diacylglycerol and the C1B domain for phorbol ester using the isolated C1A and C1B domains of PKC δ (Stahelin et al., 2004). In addition, ceramide, a lipid mediator, translocates PKC δ to the Golgi apparatus, which requires the C1B domain (Kajimoto et al., 2004). The finding that the C1B domain plays a dominant role in propofol-induced PKC δ translocation is consistent with the hypothesis that propofol, a lipophilic chemical, acts as a binding and activating agent for PKC δ, rather than inducing diacylglycerol production.

4.3. PKC translocation into the nucleus

The C2 domain deletion mutant of PKC α was translocated to the nucleus (C2deletion, C1AC2deletion, C1BC2deletion, C1ABC2deletion) (Fig. S1C and S1D, Fig. S2). PKC is translocated to the nucleus in response to specific stimuli in certain cells. For example, in NIH 3T3 fibroblasts, PKC α has been reported to be translocated from the cytoplasm to the nucleus by phorbol ester (Leach et al., 1989), in a nuclear localization signal-independent manner (Schmalz et al., 1998).

The mechanism and physiological significance of nuclear translocation of PKC are not clear at present. Further studies are required to elucidate this mechanism.

4.4 Comparison of PKC translocation induced by propofol and by PKC activators

As discussed, the domains important for propofol-induced PKC α translocation to the plasma membrane were both C1A and C1B. Similar to these results, both domains were involved in PMA-induced translocation of PKC α (Fig. S3). In contrast, the domain essential for bryostatin1-induced translocation of PKC α was C1A (Fig. S4). In addition, PKC α translocation induced by PMA and bryostatin1 does not require the C2 domain because the C1AB deletion mutant did not respond to PMA or bryostatin1 (Fig. S3D and Fig. S4D).

In the case of PKC δ, the C1B domain was essential for the induction of PKC δ translocation in propofol-, PMA- and bryostatin1-treated cells (Fig. 4, Fig. S7, and Fig. S7). However, translocation to the Golgi apparatus was specifically observed in propofol-induced mice.

Thus, PKC translocation induced by propofol was shown to be similar in some respects, but different in others, to PKC translocation induced by these PKC activators.

4.5 Inhibition of propofol-induced PKC translocation by calphostin C

We examined the effects of calphostin C, a PKC inhibitor, on propofol-induced PKC-δ translocation because the drug inhibits PKC activity by binding to the C1 domain. (Kobayashi et al., 1989). As expected, pretreatment with calphostin C suppressed propofol-induced PKCδ translocation (Fig. 5A), supporting the hypothesis that the C1 domain is involved in propofol-induced PKCδ translocation.

4.6 Phosphorylation of eNOS by propofol and its inhibition by calphostin C

It is unclear what effects of propofol are exerted via PKC activation in vivo; however, one hypothesis is that PKC activation may be involved in vasodilation through eNOS phosphorylation and subsequent NO production. Wang et al. reported that propofol induces eNOS phosphorylation and NO production, which is inhibited by PKC inhibitors bisindolylmaleimide I and staurosporine (Wang et al., 2010). In the present study, propofol-induced eNOS phosphorylation was inhibited by calphostin C. The inhibition of eNOS phosphorylation by these PKC inhibitors may prevent vasodilation caused by excessive NO production induced by propofol in vivo. Since eNOS is localized in the Golgi apparatus (Fulton et al., 2002), the suppression of PKCδ translocation to the Golgi by calphostin C may have contributed to the suppression of eNOS phosphorylation.

4.7 Propofol actions and prospects via PKC

Various PKC-mediated intracellular signal transduction pathways are involved in the regulation of neural functions (Robinson, 1991) (Huganir and Greengard, 1990). Halothane, a volatile general anesthetic, activates PKC (Hemmings et al., 1995) and induces PKC translocation (Gomez et al., 2002), suggesting that PKC is involved in its anesthetic effects. Although the anesthetic effects of propofol have been attributed to its direct action on $GABA_A$ receptors (Franks, 2006), the specific details of the mechanism are not fully understood and may be mediated by intracellular signaling changes through PKC activation.

In contrast, propofol-induced PKC activation may be responsible for its adverse effects. PRIS is a serious complication of high-dose long-term administration of propofol. The cause of PRIS is thought to be the inhibition of the respiratory chain and fatty acid oxidation in the mitochondria (Krajčová et al., 2015). Some PKCs, such as PKCε, have been reported to act on the mitochondria and induce mitochondrial dysfunction (Nowak et al., 2011). We previously revealed that propofol activates and translocates PKCε (Miyahara et al., 2018).

In addition, propofol-induced PKC activation may induce NO production via eNOS phosphorylation, which may be involved in blood pressure reduction and vascular pain during

propofol infusion. As NOS inhibitors are being developed for the treatment of primary headaches (Olesen, 2008), inhibitors of NOS or PKC may reduce the excessive local production of NO, a cause of vascular pain. Although most PKC inhibitors currently in clinical trials are ATP-competitive PKC inhibitors (Kawano et al., 2021), future drug discovery may be motivated if it becomes clear that inhibition of C1 domains would benefit the action of propofol.

5. Limitation

First, the clinical effects of propofol generally occur at blood concentration of 4-6 mg/L, which corresponds to 22-34 μM (Han et al., 2016), whereas the concentration of propofol used in this study was mostly 100 μM. However, it is possible that the local concentration at the site of propofol administration becomes higher than 22-34 μM and close to 100 μM, as well as the blood concentration when propofol is administered in high doses for long periods. Second, we have not yet confirmed whether domain deletions alter propofol-induced activation of PKC in vitro.

6. Conclusion

The domains involved in propofol-induced PKC translocation are suggested to be the C1A, C1B, and C2 domains of PKC α and the C1B domain of PKC δ. In addition, propofol induced the phosphorylation of eNOS via PKC, and its action was inhibited by calphostin C, a C1 domain inhibitor of PKC. This study revealed a part of the mechanism by which propofol induces PKC translocation.

Author statement

Soshi Narasaki: Conceptualization, Methodology, Validation, Formal analysis, Writing - Original Draft, Visualization. Soma Noguchi: Investigation, Data Curation. Tomoaki Urabe: Conceptualization, Supervision, Investigation, Funding acquisition. Kana Harada: Supervision, Investigation. Izumi Hide: Supervision, Investigation. Sigeru Tanaka: Supervision, Investigation, Conceptualization. Yuki Yanase: Investigation, Resources. Taketoshi Kajimoto: Conceptualization, Supervision. Kazue Uchida: Resources. Yasuo, M., Tsutsumi: Conceptualization, Supervision, Funding acquisition. Norio Sakai: Conceptualization, Methodology, Validation, Resources, Writing - Review & Editing, Supervision, Funding acquisition.

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Figure legends

Fig. 1. A: Structure of WT and domain-deleted mutants of PKC α used in this study.
B: Structure of WT and domain-deleted mutants of PKC δ used in this study.

Fig. 2. A: Time-series of propofol-induced translocation of WT PKC α-GFP expressed in HeLa cells. Translocation was triggered by 100 μM propofol. Pictures are representative images from 41 experiments. PKC α-GFP was translocated to the plasma membrane (1 min) and remained there. Bars indicate 10 μm.
B: Time-series of propofol-induced translocation of the C1AB domain-deleted mutant of PKC α-GFP (C1ABdeletion) expressed in HeLa cells. Translocation was triggered by 100 μM propofol. Pictures are representative images from 22 experiments. PKC was translocated to the plasma membrane (1 min) and then returned to the cytoplasm (5 min).
C: Time-series of propofol-induced translocation of C2 domain-deleted mutant of PKC α-GFP (C2deletion) expressed in HeLa cells. The translocation was triggered by 100 μM propofol. Pictures are representative images from 19 experiments. PKC was translocated to the plasma membrane (1 min), followed by to the nucleus (5 min). Bars indicate 10 μm.
D: Time-series of propofol-induced translocation of the C1AB and C2 domain-deleted mutants of PKC α-GFP (C1ABC2deletion) expressed in HeLa cells. Translocation was triggered by 100 μM propofol. Pictures are representative images from 10 experiments. PKC translocated to the nucleus (2 min). Bars indicate 10 μm.

Fig. 3. A: Patterns of translocation to the plasma membrane in WT and its domain-deleted mutants of PKC α-GFP expressed in HeLa cells. Transient (C1ABdeletion): PKC was translocated to the plasma membrane and then returned to the cytoplasm; intermediates (C1Adeletion): PKC was translocated to the plasma membrane, then some returned to the cytoplasm, and some remained at the plasma membrane; persistent (WT): PKC was translocated to the plasma membrane and remained there.
B: Percentage of translocation patterns to the plasma membrane in WT and PKC α-GFP domain-deleted mutants expressed in HeLa cells. We observed 92 cells in the WT, 22 in the C1Adeletion, 23 in the C1Bdeletion, 49 in the C1ABdeletion, 37 in the C2deletion, 10 in the C1AC2deletion, 14 in the C1BC2deletion, and 12 in the C1ABC2deletion. A higher percentage of "transient" pattern was observed in the C1 domain-deleted mutants, and almost no translocation to the plasma membrane was observed in the C1 and C2 domains-deleted mutants.
C: Percentage of translocation to the nucleus observed in WT and its domain-deleted mutants of PKC α-GFP expressed in HeLa cells. See Fig. 4B for the number of cells observed.

Translocation to the nucleus was more frequently seen in C2 domain-deleted mutants. It was always seen in C1AB and C2 domain-deleted mutant of PKC α -GFP (C1ABC2deletion).

Fig. 4. A: Time-series of propofol-induced translocation of WT PKC δ -GFP expressed in HeLa cells. Translocation was triggered by 100 μM propofol. Pictures are representative images from six experiments. PKC was translocated to the Golgi apparatus (30 sec) and then to the plasma membrane (2 min). Bars indicate 10 μm.
B: Time-series of propofol-induced translocation of the C1B domain-deleted mutant of PKC δ -GFP (C1Bdeletion) expressed in HeLa cells. Translocation was triggered by 100 μM propofol. Pictures are representative images from three experiments. PKC was not translocated. Bars indicate 10 μm.
C: Percentage of translocation to the plasma membrane observed in WT and its domain-deleted mutant PKC δ -GFP expressed in HeLa cells. We observed 10 cells in the WT, 14 in the C1Adeletion, 5 in the C1Bdeletion, and 5 in the C1ABdeletion. PKC was not translocated to the plasma membrane in C1B domain-deleted mutants.

Fig. 5. A: Time-series of propofol-induced translocation of WT PKC δ -GFP after the treatment with 2 μM calphostin C in HeLa cells. Translocation was triggered by 100 μM propofol. PKC was not translocated. Bars indicate 10 μm.
B: Effects of calphostin C on propofol-induced eNOS phosphorylation. HUVECs were treated with vehicle (control), propofol (100 μM), propofol plus calphostin C (2 μM), or calphostin C alone, followed by immunoblotting analysis using anti-eNOS and anti-peNOS antibodies.
C: Quantitative analysis of the effect of calphostin C on propofol-induced phosphorylation of eNOS. The expression levels of peNOS and eNOS were quantified using densitometry. The ratio of peNOS expression to eNOS expression (peNOS/eNOS) was determined as the eNOS phosphorylation ratio. The eNOS phosphorylation ratio was significantly enhanced by propofol exposure. However, this significant enhancement was abolished by the concurrent administration of calphostin C and propofol (*$p < 0.05$, one-way ANOVA, followed by Dunnett's post-test).

Supplementary information

Fig. S1. A: Time-series of propofol-induced translocation of C1A domain-deleted mutant of PKC α-GFP (C1Adeletion) expressed in HeLa cells. Translocation was triggered by 100 μM propofol. Pictures are representative images from 14 experiments. PKC was translocated to the plasma membrane (1 min) and then returned to the cytoplasm (10 min). Bars indicate 10 μm.
B: Time-series of propofol-induced translocation of the C1B domain-deleted mutant of PKC α-GFP (C1Bdeletion) expressed in HeLa cells. Translocation was triggered by 100 μM propofol. Pictures are representative images of 14 experiments. PKC was translocated to the plasma membrane (1 min) and then returned to the cytoplasm (5 min). Bars indicate 10 μm.
C: Time-series of propofol-induced translocation of C1A and C2 domain-deleted mutants of PKC α-GFP (C1AC2deletion) expressed in HeLa cells. Translocation was triggered by 100 μM propofol. Pictures are representative images from six experiments. PKC was not translocated. Bars indicate 10 μm.
D: Time-series of propofol-induced translocation of C1B and C2 domain-deleted mutants of PKC α-GFP (C1BC2deletion) expressed in HeLa cells. Translocation was triggered using 100 μM propofol. Pictures are representative images from six experiments. PKC was not translocated. Bars indicate 10 μm.

Fig. S2. Time-series of propofol-induced translocation of WT and its domain-deleted mutants of PKC α-GFP expressed in HUVECs. Translocation was triggered by 100 μM propofol. Pictures are representative images, and the number of experiments performed was 12 in WT, 5 in C1Adeletion, 3 in C1Bdeletion, 5 in C1ABdeletion, 4 in C2deletion, 5 in C1AC2deletion, 7 in C1BC2deletion, and 5 in C1ABC2deletion. Bars indicate 10 μm.

Fig. S3. Time-series of PMA induced translocation of WT and its domain-deleted mutants of PKC α-GFP expressed in HeLa cells. Translocation was triggered by 1 μM PMA. Bars indicate 10 μm.

Fig.S4. Time-series of bryostatin1-induced translocation of WT and its domain-deleted mutants of PKC α-GFP expressed in HeLa cells. Translocation was triggered by 500 nM bryostatin1. Bars indicate 10 μm.

Fig. S5. A: Time-series of propofol-induced translocation of C1A domain-deleted mutant of PKC δ-GFP (C1Adeletion) expressed in HeLa cells. Translocation was triggered by 100 μM

propofol. Pictures are representative images from seven experiments. Compared to the WT, PKC δ -GFP was accumulated at Golgi apparatus. Application of propofol was translocated PKC δ -GFP to the plasma membrane (2 min). Bars indicate 10 μm.
B: Time-series of propofol-induced translocation of C1A and C1B domain-deleted mutants of PKC δ -GFP (C1ABdeletion) expressed in HeLa cells. Translocation was triggered by 100 μ M propofol. Pictures are representative images from seven experiments. PKC was not translocated. Bars indicate 10 μm.

Fig. S6. Time-series of propofol-induced translocation of WT and domain-deleted mutants of PKC δ -GFP expressed in HUVECs. The translocation was triggered by 100 μ M propofol. Pictures are representative images, and the number of experiments performed was seven in WT, six in C1Adeletion, seven in C1Bdeletion, and six in C1ABdeletion. Bars indicate 10 μm.

Fig. S7. Time-series of PMA-induced translocation of WT and its domain-deleted mutants of PKC δ -GFP expressed in HeLa cells. Translocation was triggered by 1 μ M PMA. Bars indicate 10 μm.

Fig.S8. Time-series of bryostatin1-induced translocation of WT and its domain-deleted mutants of PKC δ -GFP expressed in HeLa cells. Translocation was triggered by 500 nM bryostatin1. Bars indicate 10 μm.

Fig. S9. A: Confirmation of each deletion mutant of PKC α -GFP expressed in HeLa cells analyzed by western blotting. Each mutant showed the predicted molecular size.
B: Confirmation of expression of each PKC δ -GFP deletion mutant in HeLa cells by western blotting. Each mutant showed the predicted molecular size.

Fig.1

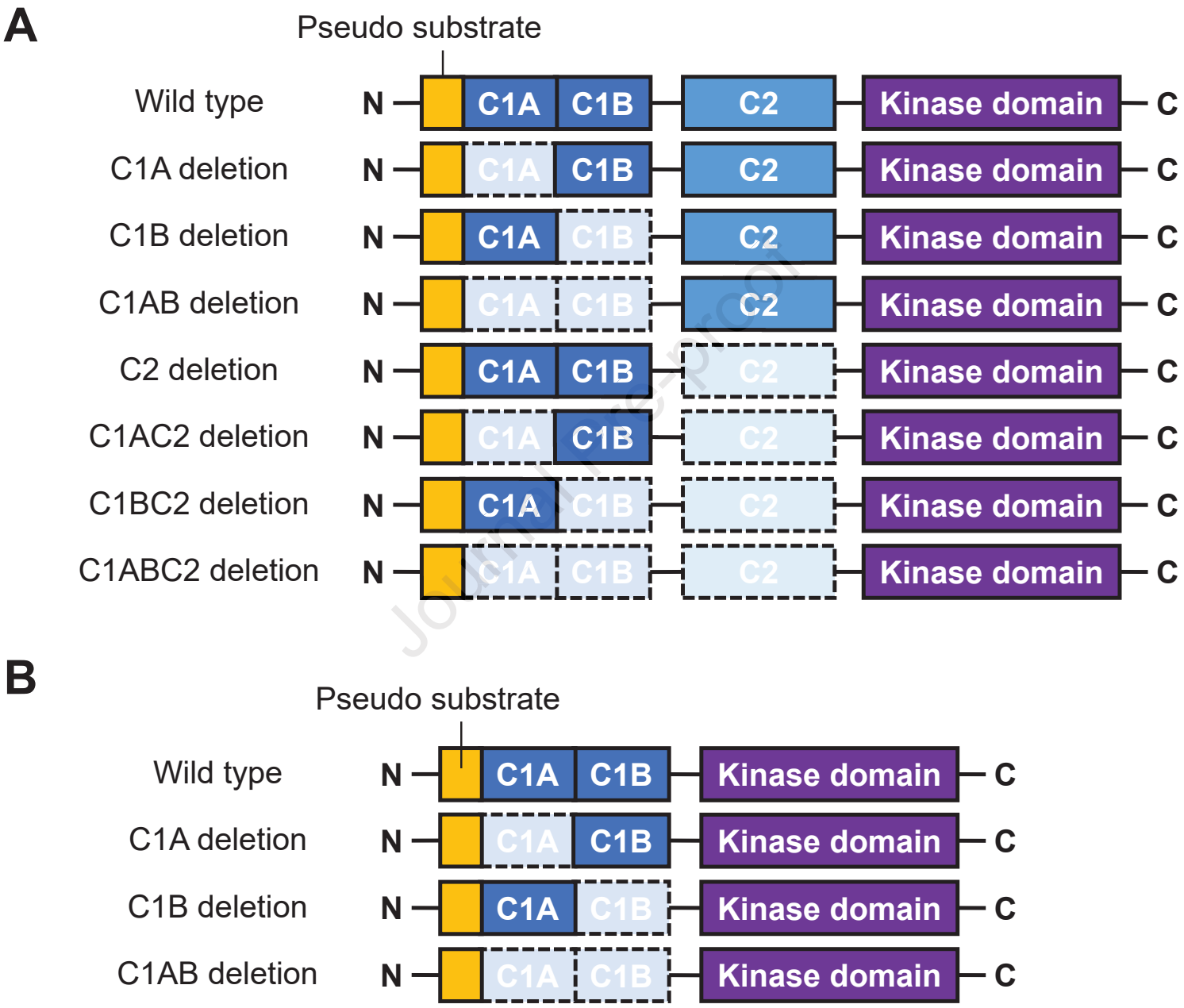

Fig.2

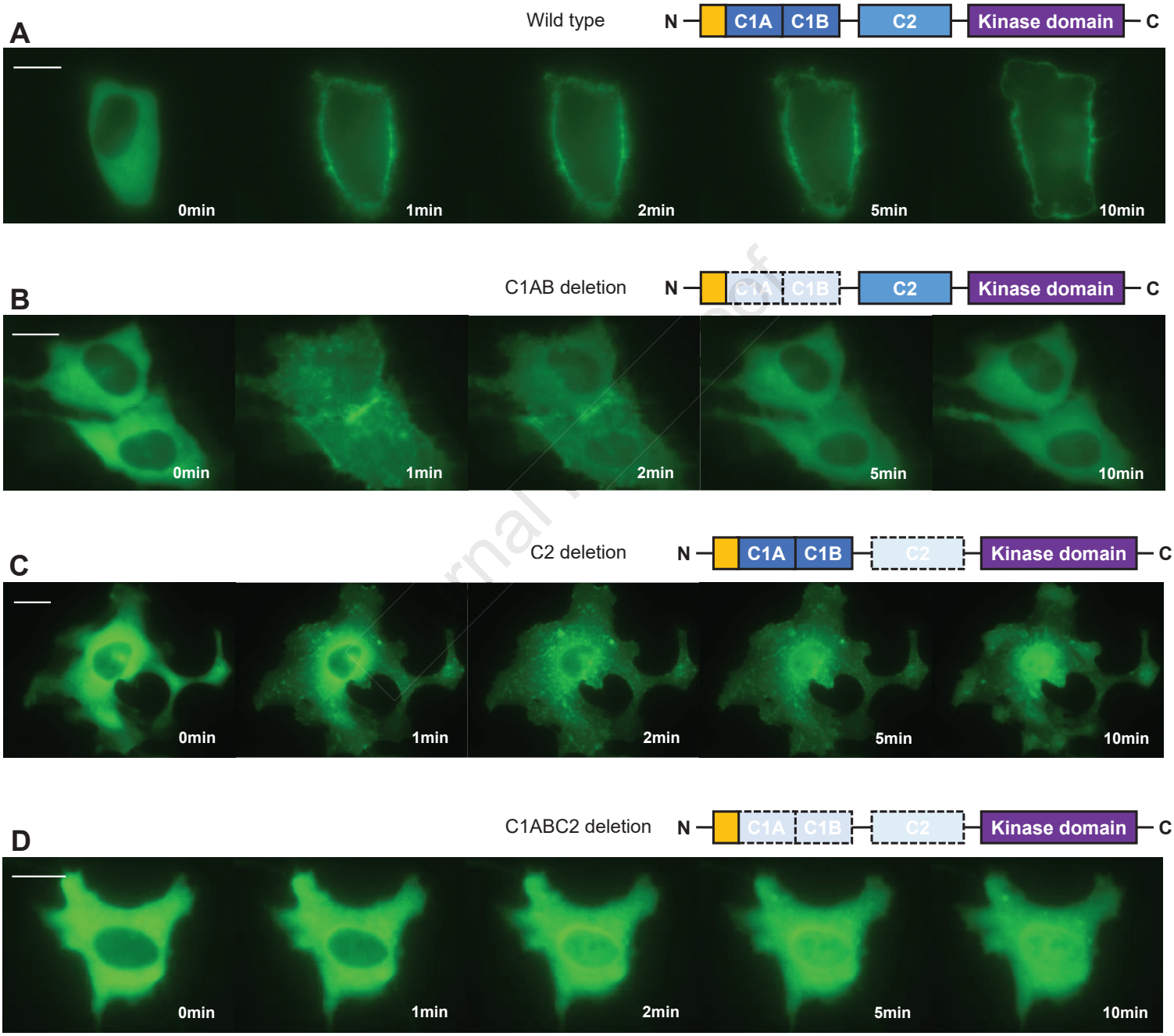

Fig.3

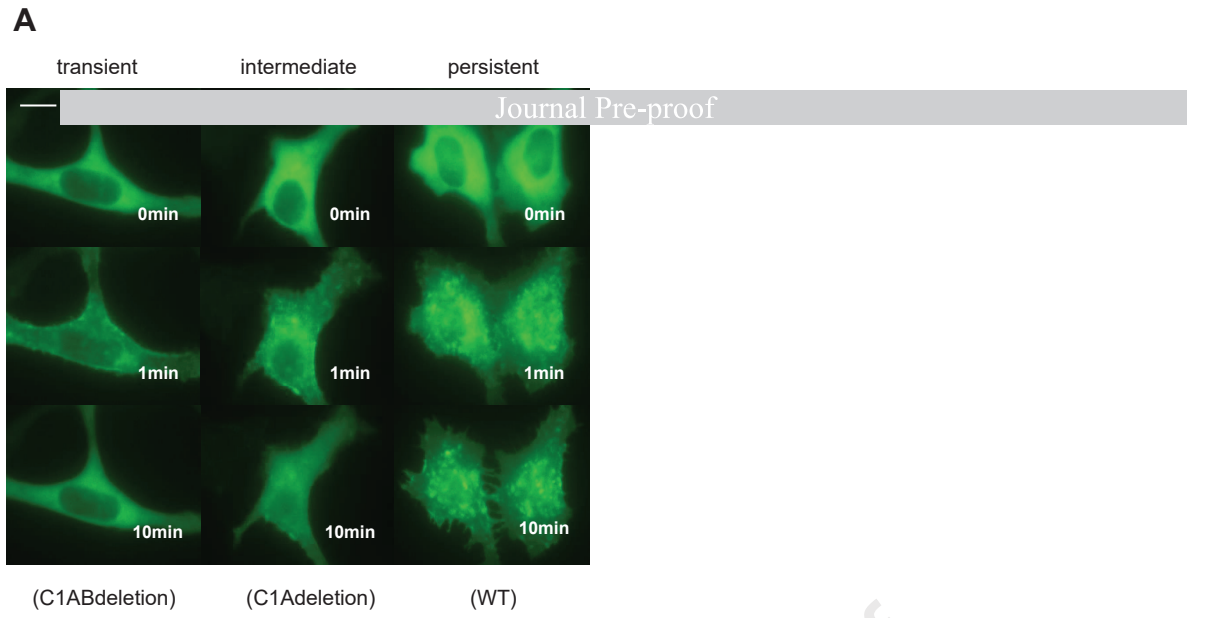
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Fig.4

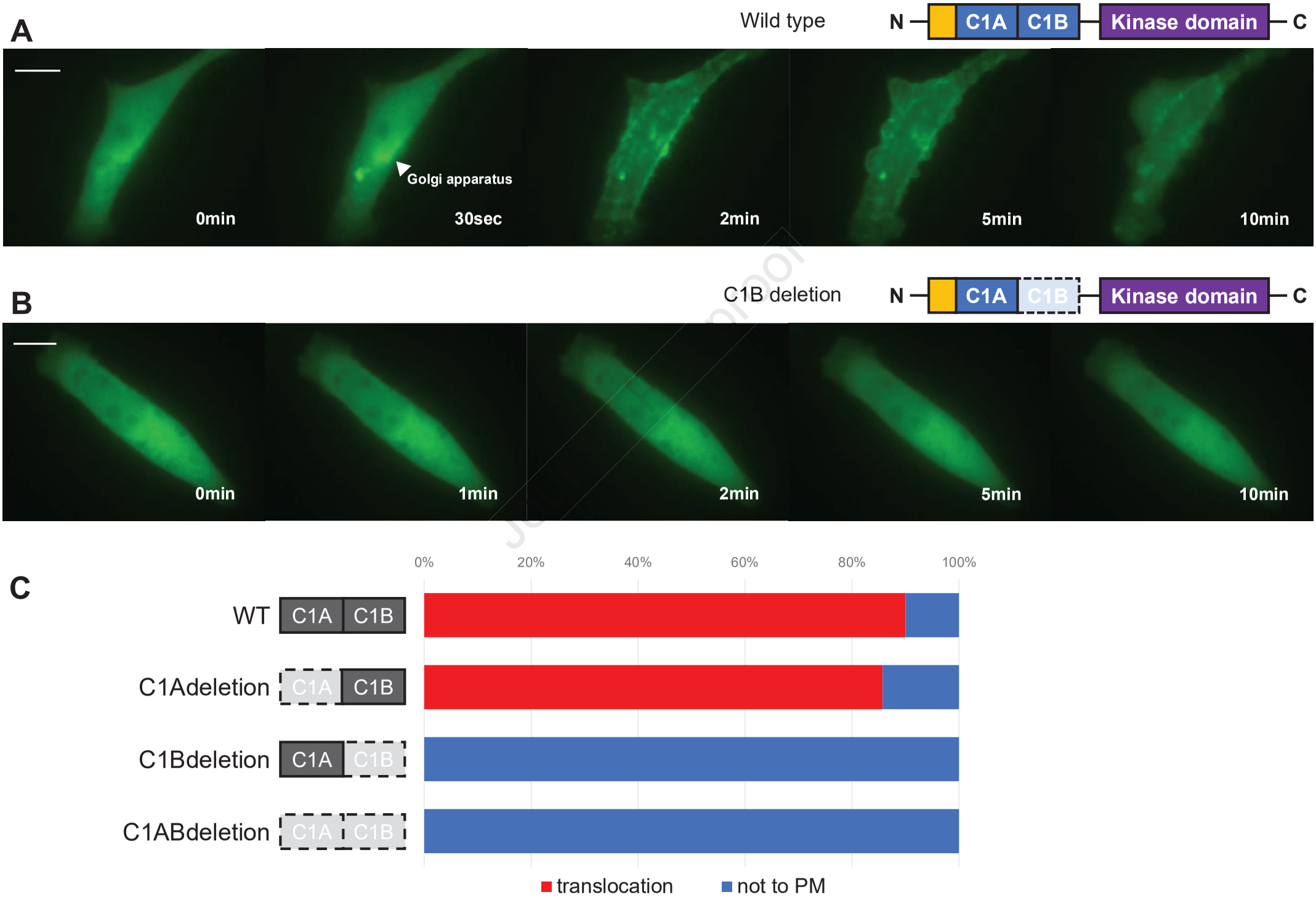
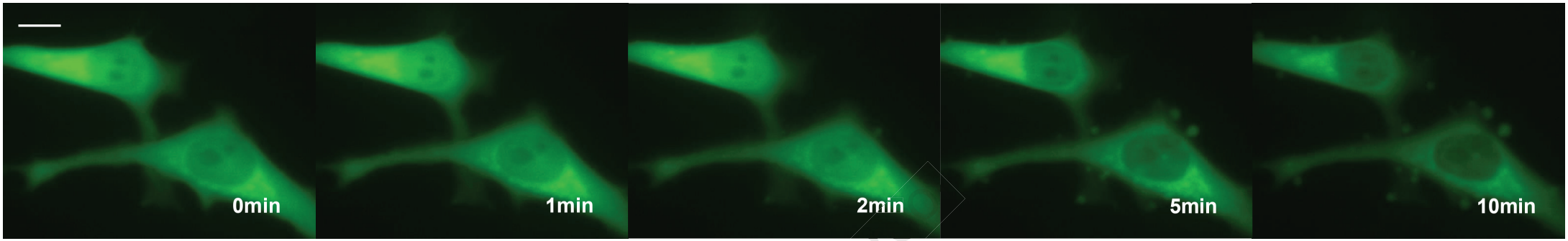
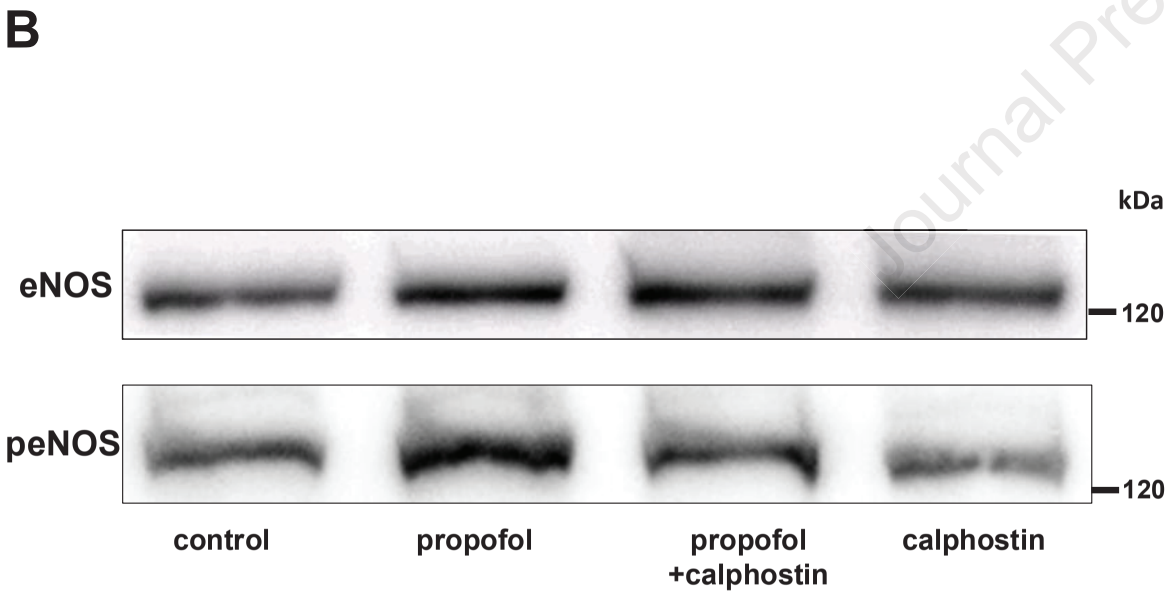
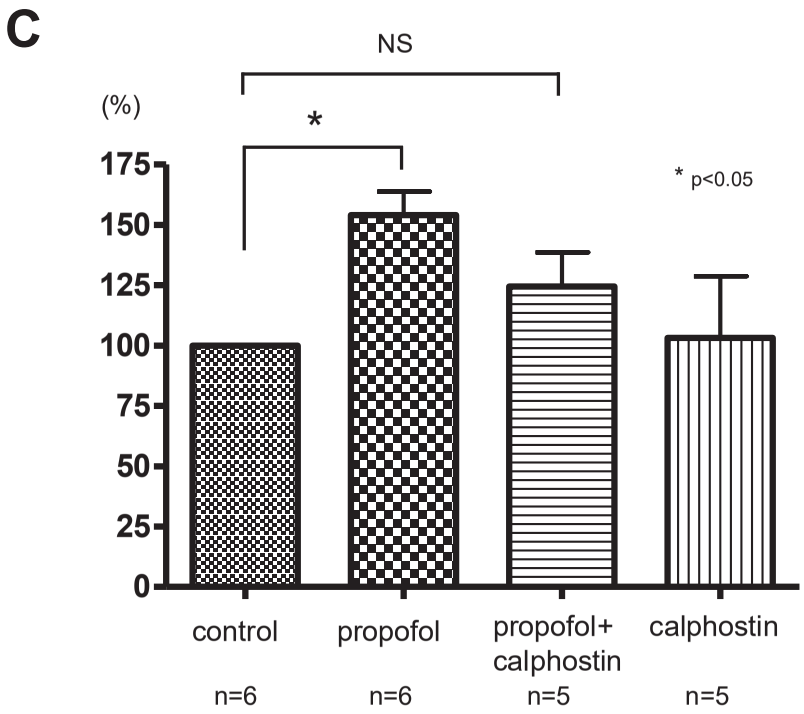


Fig.5







Highlights

Propofol. a general anesthetic, induced the translocation of PKC α and PKC δ

Both C1 and C2 domains were required for propofol-induced PKCα translocation

The C1B domain was critical for propofol-induced PKCδ translocation.

Calphostin C, a C1 domain inhibitor, abolished propofol-induced PKCδ translocation

Conflict of interest

The authors have no conflicts of interest regarding this study.