Spatial and temporal translocation of PKC α in single endothelial cell in response to focal mechanical stimulus

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19 **Summary statement**

20 In response to a focal mechanical stimulus, PKC α in an endothelial cell was initially translocated
21 toward distinct spots near or at the membrane and then accumulated at the stimulus point.

22

Abstract

We observed the kinetics of protein kinase C α (PKC α) and the intracellular Ca²⁺ wave in endothelial cells (ECs) in response to microscopic mechanical stress to investigate the effect of mechanical stress on PKC α translocation. The results show that a focal mechanical stimulus induced biphasic and directional PKC α translocation; PKC α initially translocated toward distinct spots near or at the membrane and then accumulated at the stimulus point. The low initial translocation occurred simultaneously in parallel with the increase in Ca²⁺. Initial translocation was inhibited in spite of Ca²⁺ increase when the diacylglycerol (DAG) binding domain of PKC α was inhibited, suggesting that translocation requires intracellular Ca²⁺ increase and DAG. On the other hand, high secondary translocation was delayed, occurring after the Ca²⁺ wave; however, this secondary translocation occurred even when Ca²⁺ release from the endoplasmic reticulum was inhibited, while it did not occur when the mechanosensitive (MS) channel was inhibited. These results indicated that at least Ca²⁺ influx through MS channels is required. Our results support the implication of PKC α in the Ca²⁺ signaling pathway in response to mechanical stress in ECs.

Introduction

PKC α , which belongs to the conventional PKC family, plays an important role in vascular disease regulation, such as heart failure, atherosclerosis, endothelial cell (EC) proliferation, and endothelial barrier function (Konopatskaya and Poole, 2010). The cell membrane receptors are activated by various hormones and growth factors, resulting in the hydrolyzation of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂) by phospholipase C β (PLC β) into inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). IP₃ is released into the cytosol and acts as the intracellular store for Ca²⁺ release from the endoplasmic reticulum (ER). Finally, PKC α is activated and recruited to the plasma membrane by both C1 and C2 domains, which bind DAG and Ca²⁺, respectively.

ECs line the interior surface of blood vessels; therefore their functions are also regulated by various mechanical stresses, such as mechanical stretch and shear stress and pressure from the blood stream. Accordingly, the membrane receptors are activated by these mechanical stresses, and G-protein-coupled receptors (GPCRs) play crucial roles in the mechanical stress-induced signaling pathways (Li and Xu, 2000; Li and Xu, 2007). Furthermore, the mechanical stresses directly activate tyrosine kinase-coupled receptors (RTKs) in smooth muscle cells (SMC), including platelet-derived growth factor receptor (PDGF-R) (Li et al., 2003; Tanabe et al., 2000), resulting in the activation of PKC δ (Li et al., 2003), which is one of the novel PKCs and is activated by DAG alone. PDGF is activated by mechanical stress (Hu et al., 1998; Ma et al., 1999; Tanabe et al., 2000), and it activates PKC ϵ , which is one of the novel PKCs, involving PLC γ (Moriya et al., 1996). While the intracellular Ca²⁺ increases in response to mechanical stretch via Ca²⁺-permeable mechanosensitive (MS) channel activation in human umbilical endothelial cells (Naruse and Sokabe, 1993), the inhibition of the MS channel prevents this Ca²⁺ wave from propagation. Tsukamoto et al. reported that PLC was activated in parallel with the intracellular Ca²⁺ wave in

response to a focal mechanical stimulus (Tsukamoto et al., 2010). These previous results suggested that PKC α might be activated by mechanical stress. Indeed, previously, western blot analysis revealed that PKC α in SMC was shown to be activated by membrane stretch (Li et al., 2003).

Many pharmacological studies have shown that the translocation of conventional PKC to the membrane is observed in synchrony with the Ca²⁺ wave (Lenz et al., 2002; Oancea and Meyer, 1998; Schaefer et al., 2001; Sinnecker and Schaefer, 2004). However, the kinetics of PKC in response to mechanical stress have not yet been directly observed in living cells. In this study, we observed the kinetics of PKC α and the intracellular Ca²⁺ wave in bovine vascular ECs in response to microscopic mechanical stress to investigate the effect of mechanical stress on the translocation of PKC α . Additionally, we examined the effect of Ca²⁺ on the translocation of PKC α .

Results

We first observed bovine aortic endothelial cells (BAECs) transfected with PKC α -Dronpa Green (DG) using confocal microscopy (Fig. 1). First we characterized the expression and localization of PKC α -DG in BAECs by western blot analysis and compared the results with those for wild-type PKC α (Fig. 1A and B). PKC α and DG were detected at approximately 80 kDa and 28.8 kDa, respectively, and therefore PKC α -DG was detected at 108.8 kDa. PKC α -DG was distributed homogenously based on the direct observation of DG fluorescence (Fig. 1C). PKC α was distributed in the cytosol based on the observation of anti-PKC α immunofluorescence (Fig. 1D). The colocalization of PKC α -DG fluorescence and anti-PKC α immunofluorescence demonstrated that the fusion protein was localized in the same area as PKC α (Fig. 1E). We also examined PKC α -DG translocation after the addition of ATP and PMA, which is known to activate PKC α , using confocal microscopy (Fig. 1F-I). Before the addition of ATP and PMA, PKC α -DG was

distributed homogeneously throughout the cytoplasm, and absent in the nucleus. Stimulation with ATP and PMA caused the transient translocation of PKC α -DG to all parts of the plasma membrane and its depletion in the cytoplasm.

Figure 2 shows the representative intracellular Ca²⁺ wave (A) and PKC α translocation (B) in response to a focal mechanical stimulus. The fluorescent intensity of Ca²⁺ decreased at 10 s, indicating an increase in intracellular Ca²⁺ (Fig. 2A), and gradually returned to baseline (Fig. 2A). Regarding PKC α , the intensity increased gradually until 20 s, and then decreased. Although many pharmacological studies have revealed that PKC α was translocated to the plasma membrane due to a pharmacological stimulus, such as ATP (Fig. 1), our results suggested that PKC α was translocated to the stimulus point by a microscopic mechanical stimulus. Moreover, to analyze the translocation in detail, the image before stimulation (at 0 s in Fig. 2B) was subtracted from each image (Fig. 2B). The resulting image revealed that the fluorescent intensity was distributed at the membrane of the cell and, particularly, that it was not homogenous but rather at a distinct spot near or at the membrane close to the stimulus point (Fig. 2C). Fig. 2D shows the averaged fluorescent intensity ratio as a function of time at a distinct spot near the membrane, cytosol, and stimulus point. Especially, to demonstrate the inhomogeneous translocation of PKC α , the intensity ratios at the membrane were presented at points both near and far from the stimulus point. Ca²⁺ intensity decreased gradually and reached the minimum at approximately 7.5 s after stimulus, and then gradually increased. Compared among regions, Ca²⁺ intensity changed at the stimulus point, in the cytoplasm, and then at the membrane. PKC α intensity decreased soon after the stimulus since the membrane was pushed by the mechanical stimulus, which was observed in the previous study (Tsukamoto et al., 2010). Thereafter, it increased gradually at the membrane, particularly near the stimulus point, and at the stimulus point, but not in the cytosol. At the membrane near the stimulus point, the intensity reached its maximum at 9.2 ± 0.6 s (mean $\pm$ SE), and decreased gradually and returned to base level at approximate 30 s. On the other hand, the intensity continued to increase at

the stimulus point after it had reached the maximum of the surrounding membrane, reaching a maximum at 21.7 ± 1.5 s (mean $\pm$ SE). Finally, it returned to base level at approximate 80 s. The peak intensities at the stimulus point and at the membrane near the stimulus point were 2.60 ± 0.10 and 1.62 ± 0.05 (mean $\pm$ SE), respectively. The peak time and intensity were significantly delayed and were higher at the stimulus point than at the membrane. Here we refer to PKC α translocation at a distinct spot near to the membrane and at the stimulus point as the initial and secondary translocations, respectively. The initial translocation occurred simultaneously with Ca²⁺ increase; however, the secondary translocation occurred after the Ca²⁺ activity, which is not consistent with previous pharmacological reports (Lenz et al., 2002; Oancea and Meyer, 1998; Schaefer et al., 2001; Sinnecker and Schaefer, 2004).

Figure 3 shows the representative intracellular Ca²⁺ wave (A) and PKC α translocation (B) under the inhibition of C1 domains of PKC α . PKC α was not activated and translocated toward the membrane and at stimulus point even though a Ca²⁺ wave was observed (Fig. 3C). Figure 4 shows the representative intracellular Ca²⁺ wave (A) and PKC α translocation (B) under the inhibition of C2 domains of PKC α . Figure 4C shows the resulting image of PKC α after subtracting the image before stimulation, and PKC α translocated toward the membrane. Figure 4D shows the averaged fluorescent intensity ratio as a function of time. In contrast to the inhibition of its translocation under the C1 domain, PKC α was translocated to a localized spot near membrane; however, the fluorescent intensity did not return to base line at 80 s. PKC α intensity at the stimulus point did not increase, and the secondary translocation at the stimulus point was not observed.

Figure 5 shows the representative intracellular Ca²⁺ wave (A) and PKC α translocation (B) under the inhibition of Ca²⁺ release from the ER. Ca²⁺ intensity changed in the stimulated cell only, and not in the neighboring cells. Figure 5C shows the subtracted image of PKC α using the image before stimulation, and PKC α translocated toward the membrane, particularly near the stimulus point. Figure 5D shows the averaged fluorescent intensity ratio of Ca²⁺ and PKC α -DG. Because of

the Ca^{2+} influx from the extracellular medium, a Ca^{2+} wave was observed and the maximum intensity at the stimulus point did not differ significantly from that of the control (Fig. 2). Regarding PKC α kinetics, the intensity reached its maximum (2.11 ± 0.07 , mean $\pm$ SE), (1.52 ± 0.07 , mean $\pm$ SE) at 21.9 ± 2.0 s and 7.8 ± 0.7 s at the stimulus point and at the membrane near the stimulus point, respectively. The peak time and intensity were significantly delayed and higher at the stimulus point than at the membrane, suggesting that both initial and secondary translocations were exhibited as control group (Fig. 2). Moreover, the initial translocation also occurred simultaneously with the Ca^{2+} increase. On the other hand, when the Ca^{2+} influx from the extracellular space was inhibited by the MS channel inhibitor (Fig. 6) and the removal of extracellular Ca^{2+} (Fig. 7), PKC α did not translocate at the stimulus point. particularly, both the Ca^{2+} wave and the PKC α translocation were not observed under the MS channel inhibitor (Fig. 6). The initial decrement due to the mechanical push was observed only when the MS channels were inhibited (Fig. 6). When the extracellular Ca^{2+} was removed (Fig. 7), Ca^{2+} increased because Ca^{2+} was released from the ER. However, the maximum value was not significantly lower than that at the control (Fig. 2) and under the inhibition of Ca^{2+} release from the ER (Fig. 5). Figure 7C shows the image of PKC α after subtracting the image before stimulation. PKC α intensity at the membrane near the stimulus point increased and reached its maximum (1.38 ± 0.05 , mean $\pm$ SE) only at 8.1 ± 1.3 s, indicating that the initial translocation of PKC α only was exhibited when the extracellular Ca^{2+} was removed. This initial translocation also occurred simultaneously with Ca^{2+} increase.

Discussion

PKC α is activated by both Ca^{2+} and DAG, and the hallmark of its activation is its translocation from the cytosol to the plasma membrane (Newton, 2001). In particular, PKC α is translocated homogenously to the membrane under pharmacological agents, as shown in Fig. 1. On

the other hand, previous studies using physiological agonists reported PKC α translocation is regulated by temporal and spatial changes in intracellular Ca²⁺ and that PKC α is translocated to different areas of the cell membrane depending on the Ca²⁺ influx from extracellular space (Maasch et al., 2000). In this study, we investigated PKC α translocation in the ECs in response to a microscopic mechanical stimulus using a DG fusion protein which exhibited the properties of the intact PKC α . Our results show that a focal mechanical stimulus induced a biphasic and directional PKC α translocation; namely, PKC α initially translocated toward distinct spots near or at the membrane, especially near the stimulus point, and then accumulated at the stimulus point. The low initial translocation occurred simultaneously with the increase in intracellular Ca²⁺, while the high secondary translocation was delayed, occurring after the Ca²⁺ wave, it required Ca²⁺ influx through MS channels.

A focal mechanical stimulus induced the microscopic mechanical stress on the cell membrane. Several membrane proteins, such as growth factor receptors, GPCRs, ion channels, caveolins and pumps could be candidate mechanosensors in the membrane (Li and Xu, 2007). Mechanical stress activates RTKs including PDGF-R, which regulates PKC δ , leading to the migration of smooth muscle cell (Li et al., 2003). On the other hand, G proteins, which include small molecular G proteins and heterotrimeric G proteins, could be activated by mechanical stress (Erickson et al., 2001; Gudi et al., 1998; Ishida et al., 1997; Yellowley et al., 1999) and implicated in the mechanical stress-induced signal pathways (Li and Xu, 2007). These candidate mechanosensors may be essential in biphasic PKC α translocation in response to mechanical stress. Maasch et al. demonstrated that localized Ca²⁺ determined the spatial and temporal PKC α translocation in rat vascular smooth muscle cell, and reported that thrombin acting on GPCRs induced rapid PKC α translocation; on the other hand, PDGF acting on PDGF-R in RTKs induced slow PKC α translocation (Maasch et al., 2000). Thrombin induced a PKC α translocation to spots

near the membrane at 30 s and was reversible after 2 min, while PDGF induced a translocation to the membrane within 60 s and was reversible after 8–9 min (Maasch et al., 2000). Additionally, thrombin- and PDGF-induced translocation of PKC α require different Ca²⁺ resources; thrombin-induced translocation is dependent on Ca²⁺ release from the ER and PDGF-induced translocation is dependent on Ca²⁺ influx from the extracellular space (Maasch et al., 2000). Indeed, our results show that the initial translocation—but not the secondary translocation—was observed when extracellular Ca²⁺ was removed (Fig. 7), and that secondary translocation was observed in spite of the inhibition of Ca²⁺ release from the ER (Fig. 5). Even when Ca²⁺ release from the ER was inhibited, initial translocation was observed (Fig. 5), and possibly Ca²⁺ influx from extracellular space may be substitute for Ca²⁺ release from ER. Our results suggested that both the initial and the secondary translocations require Ca²⁺ increase, and for the secondary translocation specifically, Ca²⁺ influx from the extracellular space is necessary. Since both GPCRs and RTKs are activated by mechanical stretch stresses (Hu et al., 1998; Li and Xu, 2007; Li et al., 2003), the focal mechanical stimulus in this study may activate both receptors microscopically. GPCRs and RTKs may be implicated in both the initial and the secondary translocations independently, resulting in the different activation timing of PKC α .

Previously, the translocation kinetics of PKC were parameterized by fitting a modified Bateman equation, as follows (Schaefer et al., 2001; Sinnecker and Schaefer, 2004):

$$R = R_0 + Sk_{on}/(k_{on} - k_{off})(e^{-k_{off}t} - e^{-k_{on}t}) \quad (1)$$

where R is the ratio of fluorescence intensity at the translocation region (accumulated distinct point near the membrane or stimulus point) to the non-translocation region (cytoplasm), R_0 is the ratio in resting cell just before stimulus, S is the constant, and t is time. The terms k_{on} and k_{off} are the rate constants for association and dissociation, respectively. In this study, the initial translocation was observed under the control and inhibition of Ca²⁺ release from the ER, and the removal of extracellular Ca²⁺, and the secondary translocation was observed under the control and inhibition of

Ca²⁺ release from the ER; therefore, these phenomena were quantified by fitting each translocation kinetic to Eqn 1 (Table 1). Regarding the initial translocation, there were no significant differences between the three conditions, and PKC α was activated with an increase in Ca²⁺ concentrations. PKC α repetitively translocated during Ca²⁺ oscillation with a tight coupling to the frequency of Ca²⁺ waves in human embryonic kidney cells under 20 μ M carbachol (Schaefer et al., 2001). Moreover, there were no significant differences in k_{on} and k_{off} , and the initial translocation exhibited $k_{on} \cong k_{off}$ under these conditions, with ranges of 0.17 s⁻¹–0.20 s⁻¹ and 0.16 s⁻¹–0.18 s⁻¹, respectively. These results are similar to those using pharmacological agents (Sinnecker and Schaefer, 2004), suggesting that the initial translocation in response to a focal mechanical stimulus may have the same mechanism as pharmacological agents involving GPCRs. Although extracellular Ca²⁺ was chelated by exchanging the extracellular medium for medium containing EGTA, the intracellular Ca²⁺ wave was nevertheless observed (Fig. 7B), which is consistent with the previous study of PLC activation in mechanical stimulation (Tsukamoto et al., 2010). This PLC activation was involved in the Ca²⁺ release from the ER and the Ca²⁺ wave in response to the mechanical stimulus (Tsukamoto et al., 2010), suggesting that DAG may be also produced. Furthermore, despite the Ca²⁺ wave, the initial translocation was not observed under the inhibition of the DAG-dependent C1 domain of PKC α (Fig. 3). These results suggested that this initial translocation required an increase in Ca²⁺ and the DAG-dependent C1 domain of PKC α . In contrast, there was no significant difference in k_{on} and k_{off} of the secondary translocation between two conditions, and the values were significantly smaller than those of the initial translocation, indicating that mechanics of the each translocation might be different.

In this study, in response to microscopic mechanical stress, initial translocation was toward distinct spots near or at the plasma membrane—particularly near the stimulus point—and secondary translocation was at the stimulus point. As discussed above, the initial and secondary translocations

by physiological agonists may be related to GPCR and TRK activation, respectively, and particularly PKC α in the secondary translocation might be accumulated at the membrane by TRKs activation. Conversely, Maasch et al. reported the local Ca²⁺ changes affected PKC α kinetics and proposed that the local Ca²⁺ increased the affinity of binding sites for PKC α membrane for the possible mechanism (Maasch et al., 2000). A small increase in Ca²⁺ increases the interaction between PKC α and the membrane, and a further increase would lead to PKC α activation in these areas (Luo and Weinstein, 1993; Maasch et al., 2000). Medkova and Cho proposed that the conventional PKC initially binds to the membrane surface via the Ca²⁺-dependent C2 domain, and then undergoes conformational changes that include the insertion of a C1a domain, which is one component of the C1 domain, into the membrane, resulting in the optional DAG binding (Medkova and Cho, 1999). Nalefski and Newton also proposed that the binding of Ca²⁺ to the C2 domain leads to a significantly higher affinity for membranes and stabilizes the C2 domain–membrane complex, and then searches two-dimensional space for DAG as long as the Ca²⁺ remain high (Nalefski and Newton, 2001). Based on these mechanisms, since the initial translocation is triggered by the Ca²⁺ wave across a cell, PKC α could translocate toward the membrane. However, DAG production would be lower when triggered by a focal mechanical stress than by pharmacological agonists, resulting in its translocation not being homogenous at the membrane and at localized spots near the stimulus point at the membrane. PLC activation occurred in broader cytosolic regions in parallel with the Ca²⁺ wave in response to mechanical stimulus, and particularly the high activation was localized at the membrane near the stimulus point (Tsukamoto et al., 2010). Our results show that the intensity of PKC α -DG remained relatively constant or decreased at the membrane far from the stimulus point in spite of the Ca²⁺ increase (Fig. 2D, 5D, and 7D). On the other hand, since the secondary translocation is dominated by the Ca²⁺ influx from the extracellular space, PKC α could be accumulated at the focal mechanical spot.

PKC α was translocated toward the membrane in response to mechanical stress despite the inhibition of the C2 domain, and this activation endured for longer than the other initial translocation (Fig. 4). Previous studies report that mechanical stress activates PLC (Tsukamoto et al., 2010) and may produce DAG, and deletion of the C2 domain of PKC α abolishes the translocation by ionomycin (Maasch et al., 2000). Therefore, this mechanism should not be the same as the initial translocation, in which both Ca²⁺ and DAG may be involved, but may instead be dominated only by DAG. PKC α is activated directly by phorbol ester, which is analogous to DAG, and this mechanism might be different from the initial translocation (Medkova and Cho, 1999). As the different mechanism, reversible and irreversible activations of PKC were proposed. Based on this mechanism, phorbol ester induced sustained activation of PKC by forming an irreversible PKC membrane complex (Kazaniets et al., 1992). On the other hand, DAG acted through a reversible complex (Bazzi and Nelsestuen, 1989) and its activation was short-lived because DAG was rapidly metabolized (Newton, 2001); both, however, caused a dramatic increase in the affinity of PKC for membranes, serving as “molecular glue” to recruit PKC to the membrane (Newton, 2001). The resulting, activation time in response to mechanical stress may be longer than calcium-related initial translocation.

Initial and secondary translocations exhibited $k_{on} \cong k_{off}$. Sinnecker and Schaefer proposed that $k_{on} \cong k_{off}$ implies a lower DAG concentration, while $k_{on} > k_{off}$ implies a higher DAG concentration, and DAG binding delays the dissociation (Sinnecker and Schaefer, 2004). Regarding the initial translocation, as discussed above, DAG might be produced through PLC activation (Tsukamoto et al., 2010). The secondary translocation was inhibited when the C1 domain was inhibited, suggesting that secondary translocation may require DAG. However, since DAG is rapidly metabolized (Newton, 2001), it remains unclear whether secondary translocation requires DAG and whether DAG is also accumulated at the mechanical stress spot. Once PKC binds to the membrane via the C2 domain, it inserts a C1a domain for searching for DAG and releases the

pseudo-substrate region from the active site, resulting in PKC α activation (Medkova and Cho, 1999). Therefore, the C2 domain of PKC α alone can bring PKC molecules to the membrane, and the membrane binding of the C1 domain might be primed by the membrane binding of the C2 domain (Kazanietz et al., 1992; Medkova and Cho, 1999).

From our results, it also remains unclear whether GPCRs and RTKs are activated directly by a focal mechanical stress. Previous studies reported that ATP release into extracellular spaces is induced by a mechanical stretch (Grygorczyk et al., 2013; Takahara et al., 2014). Takada et al. reported that this extracellular ATP is released through mechanosensitive hemichannels and acts as an autocrine mediator to activate GPCRs (Takada et al., 2014). On the other hand, Hayakawa et al. demonstrated that the increase in tension of actin stress fibers could activate MS channels and the actin skeleton acted as a force-transmitting and-focusing device between integrin and MS channels using ECs (Hayakawa et al., 2008). Indeed, in this study, the Ca²⁺ wave and PKC α translocation were not observed when MS channels were inhibited (Fig. 6), suggesting that GPCRs and RTKs might be activated by a focal mechanical stress via MS channel activation.

In this study, a focal mechanical stimulus induced the biphasic and directional PKC α translocation in single ECs. In its initial translocation that may involve the GPCRs signal pathway, PKC α is translocated toward the membrane in parallel with the Ca²⁺ wave. In secondary translocation, which may involve the RTKs signal pathway, PKC α was accumulated to the focal stimulus point after the Ca²⁺ wave. However, Ca²⁺ influx through MS channels from extracellular space, not Ca²⁺ release from ER, is required. PKC α is involved in several physiological functions, in the migration, proliferation, and angiogenesis of ECs (Bokhari et al., 2006; Newton, 2001; Wang et al., 2002); therefore, PKC α activation in response to focal mechanical stress may be helpful in understanding mechanotransduction signaling.

Materials and Methods

cDNA construction

Hybrid cDNAs encoding fusions of bovine PKC α (Plasmid 10805; Invitrogen) and DG (pDG1-MN1, AM-V0073; Medical & Biological Laboratories Co., LTD, JAPAN) were inserted into the multiple cloning site of the pcDNATM3.1(+) (V790-20; Invitrogen) mammalian expression vector. We created the linker that connected PKC α to DG (Wagner et al., 2000). cDNAs encoding PKC α , DG, and the linker were amplified by polymerase chain reaction using the following primers: 5'-PKC α , 5'-ACCCAAGCTGGCTAGCACCATGGCTGAC-3'; 3'-PKC α , 5'-GTCGACTGCAGAATTCGCCCTTGTTTACTACCG-3'; 5'-DG, 5'-CCCGGGATCCACCGACCGGTCGCCACCATGGTGAGTGTGATT-3'; 3'-DG, 5'-GCCCTCTAGACTCGAGACTTGGCCTGCCTCGGCAG -3'; 5'-Linker, 5'-TAAGAATTCTGCAGTCGACGG-3'; and 3'-Linker, 5'-TAATATGCGGCCGCTATAACC-3'. The NheI/EcoRI-digested PKC α fragment and the AgeI/XhoI-digested DG fragment were ligated into the NheI/XhoI-digested vector, generating the PKC α -DG fusion construct. All constructs were confirmed by DNA sequencing (Bio Matrix Research, Inc., Kashiwa, Chiba, Japan).

Cell culture and transfection

BAECs (Toyobo Co., Ltd., Osaka, Japan) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO™; Thermo Fisher Scientific, Waltham, MA, USA), containing 1% Antibiotic-Antimycotic (10000 U/ml penicillin, 10000 μ g/ml streptomycin, and 25 μ g/ml amphotericin B, GIBCO™; Thermo Fisher Scientific) and 10% (v/v) fetal bovine serum (Biological Industries, Kibbutz Beit-Haemek, Israel), in humidified incubators containing 5% CO₂ at 37 °C.

Prior to transfection, BAECs were seeded in a 35-mm glass-bottomed dish and cultured to 70% confluence. BAECs adherent to the glass-bottomed dishes were washed twice with opti-MEM (GIBCO™; Thermo Fisher Scientific). We changed DMEM for 1 ml of opti-MEM, and the BAECs were cultured for 14–16 h. Two microliters of DNA (1 μ g/ μ l) in 100 μ l of opti-MEM were mixed

with 4 μ l of HilyMax (Dojindo Laboratories, Kumamoto, Japan). This solution, including the construct DNA, was then added to the BAECs adherent to the glass-bottomed dish, together with 1 ml of opti-MEM, and PKC α -DG was transfected into the BAECs. We used BAECs (Passages 6–10) grown to confluence for experiments 2 days after transfection.

Immunoblotting

After the protein concentration was measured using the Lowry method, equivalent amounts of protein were prepared and separated on a 7.5% polyacrylamide-sodium dodecyl sulfate gel and transferred to an Immun-Blot® polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were washed three times with Tris-buffered saline (TBS; 0.02 M Tris and 0.5 M NaCl; Bio-Rad Laboratories, Inc.) containing 0.1% (v/v) Tween-20 (Bio-Rad Laboratories, Inc.). The membranes were successively incubated, first with blocking buffer containing TBS, 0.1% (v/v) Tween-20, and 3% (w/v) Albumin (Nacalai Tesque, Inc., Kyoto, Japan) for 1 h at room temperature. The next incubation was conducted with the primary antibodies anti-PKC α Clone M4 (EMD Millipore Corp., Billerica, MA, USA) and anti-DG (MBL Laboratories Co, JAPAN) at a dilution of 1:5000 for 1 h at room temperature. After washing twice with TBS containing 0.1% (v/v) Tween-20, a third incubation was performed with the secondary antibody anti-mouse Immunoglobulin G (IgG) biotin (eBioscience, Inc., San Diego, CA, USA) at a dilution of 1:5000 for 1 h at room temperature. Thereafter, the membranes were probed with Pierce® High-Sensitivity Streptavidin-HRP (Thermo Fisher Scientific) at a dilution of 1:5000 for 1 h at room temperature. Finally, the membranes were incubated with Luminata Forte Western HRP (Merck KGaA) for 1 min at room temperature.

Characterization of the PKC α -DG in BAECs

For immunocytochemistry, transfected BAECs were fixed with 4% paraformaldehyde for 10 min. After washing twice with phosphate-buffered saline (PBS), BAECs were permeabilized with PBS containing 0.1% (v/v) Triton-X for 5 min. After washing three times with PBS, the preparation

was incubated for 1 h at room temperature with mouse anti-PKC α Clone M4 (Clontech Laboratories, Inc.) at a dilution of 1:200. The preparation was washed three times with PBS, and then exposed to the secondary antibody (Alexa Fluor 568-conjugated anti-mouse IgG, 1:200; Life Technologies, Carlsbad, CA, USA) for 1 h. Then, the preparation was washed three times with PBS, and observed in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline (HBS; 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 10 mM D-glucose, and 15 mM HEPES). Fluorescent images were captured by confocal microscopy (Digital Eclipse C1; Nikon Corp., Tokyo, Japan) using a 60X oil-immersion lens (S-Fluor, NA = 1.4; Nikon Corp.). Confocal images of DG were obtained by excitation with a sapphire laser at 488 nm and using a reflection short-pass 480-nm filter for GFP. Confocal images of Alexa Fluor 568 were obtained by excitation with a helium neon laser at 543 nm and using a reflection 545-nm filter. Emission wavelengths were detected using a 515 ± 15 nm filter for GFP and a 605 ± 30 nm filter for Alexa Fluor 568.

For pharmacological study, the translocation of PKC α -DG to the cell membrane was observed in the addition of 200 μ M ATP and 500 μ M PMA (Sigma-Aldrich Corp., St. Louis, MO, USA) using confocal microscopy (Digital Eclipse C1) with a 60X oil-immersion lens (S-Fluor, NA = 1.4). Confocal images of PKC α -DG were obtained by excitation with a sapphire laser at 488 nm and using a reflection short-pass 480 nm filter. The emission wavelength was detected using a 515 ± 15 nm filter.

Mechanical stimulation

The mechanical stimulation of BAECs was performed on a single cell using a fine glass micropipette. The tip of the pipette was pulled so as to be less than 3 μ m in diameter (P-97; Sutter Instrument Company, Novato, CA, USA). To prevent rupture of the plasma membrane, the tip of the micropipette was heat-polished. The micropipette was fixed in a micromanipulator (MMO-203; Narishige Group, Tokyo, Japan). The tip of the micropipette was softly attached to the plasma

membrane surface. Micropipette indentation depth was approximately 1 μm –3 μm , a depth which was estimated using the scale on the manual micromanipulator.

Simultaneous measurement of changes in intracellular Ca^{2+} concentration and PKC α –DG translocation

BAECs were cultured in a 35-mm glass-bottomed dish to 100% confluence for 48 h after transfection. In this study, in addition to PKC α kinetics, we examined the intracellular Ca^{2+} wave in response to the microscopic mechanical stress by observing the decrease in the fluorescent intensity of the Ca^{2+} indicator Fura-2–acetoxymethyl ester (Fura-2–AM; Invitrogen, Carlsbad, CA, USA). Transfected BAECs were washed twice with HBS and then loaded with 5 μM Fura-2–AM in HBS for 30 min at room temperature. After washing twice with HBS, the transfected and Fura-2–AM-loaded cells were placed on the stage of an inverted microscope (Eclipse TE2000-S; Nikon Corp.) fitted with a 40X oil-immersion lens (S-Fluor, NA = 1.30; Nikon Corp.). In the light path, a 500 nm dichroic mirror and a 535 ± 45 nm emission filter were used. The excitation light was emitted by a xenon lamp (C7773; Hamamatsu Photonics K.K., Hamamatsu, Shizuoka Japan), and the wavelength was filtered using the AquaCosmos system (version 2.61; Hamamatsu Photonics K.K.). The Fura-2–AM was excited with 390 nm, and the PKC α –DG was excited with 490 nm. To monitor the fluorescence of both intracellular Ca^{2+} and PKC α –DG simultaneously, the excitation wavelength shifted at 148 ms intervals. The fluorescent emission from the sample was amplified with an image intensifier (C8600-03; Hamamatsu Photonics K.K.), detected by a charge-coupled device camera (C6790; Hamamatsu Photonics K.K.), and recorded on a personal computer. The microscope stage was maintained at 37 °C by enclosure in thermal insulation material.

In the inhibition experiments, the following inhibitors were used: 20 nM Bryostatin (Sigma-Aldrich Corp., St. Louis, MO, USA) for the DAG-dependent C1 domain for PKC α ; and 10 μM Go6976 (Sigma-Aldrich Corp., St. Louis, MO, USA) for inhibiting the Ca^{2+} -dependent C2 domain of PKC α . Additionally, to investigate the effect of Ca^{2+} on PKC α kinetics, 200 nM Thapsigargin

(Sigma-Aldrich Corp., St. Louis, MO, USA) for inhibition of Ca^{2+} release from the ER, and 20 μM Gadolinium Chloride Hexahydrate (Gd^{3+} ; Wako pure chemical industries, Ltd, JAPAN) for inhibiting the MS channel were loaded onto the BAECs after Fura-2-AM loading. Gd^{3+} and other inhibitors were dissolved in HBS and DMSO, respectively. These inhibitors were loaded for 30 min for Gd^{3+} and Go6976, for 1 hour for Thapsigargin, and for 6 hours for Bryostatin, and then washed with HBS. Furthermore, for the removal of extracellular Ca^{2+} in HBS, HBS was exchanged with HBS containing EGTA (140 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 10 mM D-glucose, 15 mM HEPES, and 200 μM EGTA).

PKC α -DG and intracellular Ca^{2+} fluorescent intensity were evaluated according to the intensity ratio in the region of interest (ROI) with a diameter of 2 μm . The fluorescent intensity ratio was defined as the fluorescent intensity at any time divided by the time-averaged intensity before stimulation during about 3 s (20 images). In this study, we measured the intensity of PKC-DG and Ca^{2+} at the stimulated point, at the cell membrane nearby, and in the cytoplasm, respectively. Regarding the cell membrane nearby, the increase in PKC-DG intensity was not homogenous throughout the membrane. Therefore, the average image before stimulation was subtracted from all the images, and the ROI was defined as the specific spot with the increasing intensity extending to a maximum depth of 4 μm from the edge of the cell. Regarding the cytoplasm, the ROI was defined as the area between the stimulation spot and the specific membrane spot.

Statistical analysis

Statistical analyzes were performed using analysis of variance. Multiple comparisons were performed using Dunnett's test. Differences were considered significant at a p value < 0.05. The number of glass-bottomed culture dishes and cells were denoted by N and n, respectively.

Conflict of Interest Statement

No conflicts of interest, financial or otherwise, are declared by the authors.

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Figure legends

Table 1 The association (k_{on}) and dissociation (k_{off}) of initial and secondary translocations of PKC α . Data are averaged values $\pm$ S.E. $^{\#}P < 0.05$ vs initial translocation.

Fig. 1 Characterization of the PKC α -DG expressed in bovine aortic endothelial cells. (A) Immunoblots of cell lysates from untransfected and PKC α -DG- BAECs probed with an antibody against PKC α . (B) Immunoblots of cell lysates from untransfected and PKC α -DG- BAECs probed with an antibody against DG. (C) PKC α -GFP. (D) Anti-PKC α . (E) Colocalization. (F and G) PKC α -GFP localization before (F) and 15 s after (G) stimulation with ATP. (H and I) PKC α -GFP localization before (H) and 15 min after (I) stimulation with PMA.

Fig. 2 The Ca $^{2+}$ wave and PKC α kinetics under the mechanical stimulus. The representative intracellular Ca $^{2+}$ wave (A) and PKC α translocation (B and C). (C) is normalized PKC α -DG translocation, which is obtained by subtracting the images at $t = 0$ from every time-series image in (B). The cell geometry and stimulus point are indicated by the dotted line and “x”, respectively. The intensities of PKC α -DG and Ca $^{2+}$ are shown using a pseudo color scale. (D) is the fluorescent intensity ratio of calcium and PKC α -DG at the stimulus point (SP), membrane (M), and cytoplasm (C) (averaged values $\pm$ S.E., N = 9, n = 17). PKC α translocations at M were presented near and far from SP, respectively. The cells are stimulated at 3 s (arrow).

Fig. 3 The Ca $^{2+}$ wave and PKC α kinetics under the mechanical stimulus when the C1 domain of PKC α was inhibited. The representative intracellular Ca $^{2+}$ wave (A) and PKC α translocation (B). The cell geometry and stimulus point are indicated by the dotted line and “x”, respectively. The intensities of PKC α -DG and Ca $^{2+}$ are shown using a pseudo color scale. (C) shows the fluorescent intensity ratio of calcium and PKC α -DG at the stimulus point (SP), membrane (M), and cytosol (C) (averaged values $\pm$ S.E., N = 6, n = 20). The cells are stimulated at 3 s (arrow).

Fig. 4 The Ca^{2+} wave and PKC α kinetics under the mechanical stimulus when the C2 domain of PKC α was inhibited. The representative intracellular Ca^{2+} wave (A) and PKC α translocation (B and C). (C) is normalized PKC α –DG translocation, which is obtained by subtracting the images at $t = 0$ from every time-series image in (B). The cell geometry and stimulus point are indicated by the dotted line and “×”, respectively. The intensities of PKC α –DG and Ca^{2+} are shown using a pseudo color scale. (D) is the fluorescent intensity ratio of calcium and PKC α -DG at the stimulus point (SP), membrane (M), and cytoplasm (C) (averaged values $\pm$ S.E., $N = 5$, $n = 15$). PKC α translocations at M were presented near and far from SP, respectively. The cells are stimulated at 3 s (arrow).

Fig. 5 The Ca^{2+} wave and PKC α kinetics under the mechanical stimulus when Ca^{2+} release from the ER was inhibited. The representative intracellular Ca^{2+} wave (A) and PKC α translocation (B and C). (C) is normalized PKC α –DG translocation, which is obtained by subtracting the images at $t = 0$ from every time-series image in (B). The cell geometry and stimulus point are indicated by the dotted line and “×”, respectively. The intensities of PKC α –DG and Ca^{2+} are shown using a pseudo color scale. (D) shows the fluorescent intensity ratio of calcium and PKC α -DG at the stimulus point (SP), membrane (M), and cytosol (C) (averaged values $\pm$ S.E., $N = 5$, $n = 13$). PKC α translocation at M was presented near and far from SP, respectively. The cells are stimulated at 3 s (arrow).

Fig. 6 The Ca^{2+} wave and PKC α kinetics under the mechanical stimulus when the MS channel was inhibited. The representative intracellular Ca^{2+} wave (A) and PKC α translocation (B). The cell geometry and stimulus point are indicated by the dotted line and “×”, respectively. The intensities of PKC α –DG and Ca^{2+} are shown using a pseudo color scale. (C) shows the fluorescent intensity ratio of calcium and PKC α -DG at the stimulus point (SP), membrane (M), and cytoplasm (C) (averaged values $\pm$ S.E., $N = 5$, $n = 16$). The cells are stimulated at 3 s (arrow).

Fig. 7 The Ca^{2+} wave and PKC α kinetics under the mechanical stimulus when extracellular Ca^{2+} was removed. The representative intracellular Ca^{2+} wave (A) and PKC α translocation (B and C).

483 (C) is the normalized PKC α –DG translocation, which is obtained by subtracting the images at $t = 0$
 484 from every time-series image in (B). The cell geometry and stimulus point are indicated by the
 485 dotted line and “×”, respectively. The intensities of PKC α –DG and Ca²⁺ are shown using a pseudo
 486 color scale. (D) shows the fluorescent intensity ratio of calcium and PKC α -DG at the stimulus point
 487 (SP), membrane (M), and cytoplasm (C) (averaged values $\pm$ S.E., N = 5, n = 16). PKC α
 488 translocations at M were presented near and far from SP, respectively. The cells are stimulated at 3
 489 s (arrow).

Table 1 The association (k_{on}) and dissociation (k_{off}) of initial and secondary translocations of PKC α .

Data are averaged values $\pm$ S.E. $^{\#}P < 0.05$ vs initial translocation.

	Initial translocation		Secondary translocation	
	k_{on}	k_{off}	k_{on}	k_{off}
Control	0.180 ± 0.012	0.181 ± 0.011	$0.084 \pm 0.006^{\#}$	$0.084 \pm 0.006^{\#}$
Inhibition of Ca $^{2+}$ release from ER	0.168 ± 0.010	0.164 ± 0.011	$0.082 \pm 0.008^{\#}$	$0.087 \pm 0.010^{\#}$
Removal of extracellular Ca $^{2+}$	0.197 ± 0.015	0.161 ± 0.022	-	-

496 Fig. 1 Characterization of the PKC α -DG expressed in bovine aortic endothelial cells.

497 Fig. 2 The Ca^{2+} wave and PKC α kinetics under the mechanical stimulus.

498 Fig. 3 The Ca²⁺ wave and PKCα kinetics under the mechanical stimulus when the C1 domain of
499 PKCα was inhibited.

500

Fig. 4 The Ca²⁺ wave and PKCα kinetics under the mechanical stimulus when the C2 domain of PKCα was inhibited.

504 Fig. 5 The Ca^{2+} wave and PKC α kinetics under the mechanical stimulus when Ca^{2+} release from
505 the endoplasmic reticulum (ER) was inhibited.
506

Fig. 6 The Ca²⁺ wave and PKCα kinetics under the mechanical stimulus when the MS channel was inhibited.

511 Fig. 7 The Ca^{2+} wave and PKC α kinetics under the mechanical stimulus when the extracellular
512 Ca^{2+} was removed.