Biphasic and directed translocation of protein kinase Cα inside cultured endothelial cells before migration

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ABSTRACT

Mechanical wounding of an endothelial monolayer induces an immediate Ca^{2+} wave. Several hours later, the denuded area is covered by endothelial cells (ECs) that migrate to the wound. This migration process is closely related to protein kinase Cα (PKCα), a Ca^{2+}-dependent protein that translocates from the cytosol to the cell membrane. Because the cells adjacent to the wounded area are the first to migrate into the wound, we investigated whether a mechanical wound immediately induces PKCα translocation in adjacent cells. We monitored Ca^{2+} dynamics and PKCα translocation simultaneously using fluorescent microscopy. For this simultaneous observation, we used Fura-2–acetoxymethyl ester to visualize Ca^{2+} and constructed a green fluorescent protein-tagged fusion protein to visualize PKCα. Mechanical wounding of the endothelial monolayer induced an immediate Ca^{2+} wave in cells adjacent to the wounded cells before their migration. Almost concurrently, PKCα in the neighboring cells translocated to the cell membrane, then accumulated at the periphery near the wounded cell. This report is the first description of this biphasic and directed translocation of PKCα in cells before cell migration. Our results may provide new insights into the directed migration of ECs.

1. Introduction

Endothelial cells (ECs) line the inner surface of blood vessels. It has been established that ECs have many functions in the regulation of the vascular system and that these functions are modulated by mechanical forces [1]. In addition, ECs exhibit wound repair capabilities. After an endothelial monolayer is denuded by a mechanical wound, ECs migrate to the denuded area; after several hours, the denuded area is covered by ECs [2].

Protein kinase Cα (PKCα) is activated by an increase in intracellular Ca^{2+}, and is involved in several physiological functions [3]. Bokhari et al. [2] found that PKCα is important for the cell migration after mechanical wounding. They also found that after an EC monolayer is denuded by mechanical wounding, ECs migrate to cover the denuded area. Inhibitors of PKCα attenuate this migration. Wang et al. [4] investigated EC proliferation and migration using antisense PKCα. They showed that antisense PKCα reduces EC migration in a scratch wounding assay and reduces the number of EC tubes formed in a collagen gel assay. These results suggested that PKCα is implicated in the

migration, proliferation, and angiogenesis of ECs.

Immediately after mechanical stimulation and wounding, but before cell migration, an increase in intracellular Ca^{2+} levels propagates from cells at the edge of the denuded area to cells distant from the wound [5]. This phenomenon is termed a Ca^{2+} wave. Since PKCα is a Ca^{2+}-dependent protein, the relationship between EC migration/proliferation and PKCα activation has been investigated several hours after mechanical wounding. Interestingly, it should be noted that preceding the cell migration, a Ca^{2+} wave is required for the subsequent motility of ECs [5]. Nevertheless, to date, no studies have examined changes in PKCα activation before the migration.

The hallmark of PKCα activation is its translocation from the cytosol to the plasma membrane [3]. Several researchers have investigated the translocation of PKCα and its relationship with intracellular Ca^{2+} levels in living cells exposed to chemical substances such as ATP [6] using PKCα tagged with green fluorescent protein (GFP). The translocation of PKCα has been observed immediately after chemical stimulation, and additionally, the intracellular Ca^{2+} wave has been observed immediately after mechanical stimulation or micropipette-mediated

wounding [7–9]. Based on these results, we hypothesized that PKCα is activated and translocated during the Ca^{2+} wave immediately after the mechanical wounding, but before the migration. In particular, when the cell is wounded mechanically, a Ca^{2+} wave is rapidly induced in its neighboring cells and their subsequent motility is initiated and regulated by this Ca^{2+} wave [5]. Thus, in this study, we wounded and killed single cells microscopically by a mechanical poke and examined the Ca^{2+} wave and PKCα kinetics in the neighboring cells before cell migration to the denuded area using bovine aortic endothelial cells (BAECs).

2. Methods

2.1. cDNA construction

Hybrid cDNAs encoding fusions of bovine PKCα (Plasmid 10805; Invitrogen) and GFP (pAcGFP1-Tubulin, 632488; Clontech Laboratories, Inc., Mountain View, CA, USA) were inserted into the multiple cloning site of the pcDNA™3.1(+) (V790-20; Invitrogen) mammalian expression vector. We used the method of Wagner *et al.* [10] to create the linker that connected PKCα to GFP. cDNAs encoding PKCα, GFP, and the linker were amplified by the polymerase chain reaction using the following primers: 5′-PKCα, 5′-ACCCAAGCTGGCT-AGCACCATGGCTGAC-3′; 3′-PKCα, 5′-GTCGACTGCAGAATTCGCCCTT-GTTTACTACCG-3′; 5′-GFP, 5′-TTAAATAACCGGTCGCCACCATGG-3′; 3′-GFP, 5′-AAACACTCGAGATCAGAGTCCGG-3′; 5′-Linker, 5′-TAAGA-ATTCTGCAGTCGACGG-3′; and 3′-Linker, 5′-TAATATGCGGCCGCTAT-ACC-3′. The *NheI/EcoRI*-digested PKCα fragment and the *AgeI/XhoI*-digested GFP fragment were ligated into the *NheI/XhoI*-digested vector, generating the PKCα–GFP fusion construct. All constructs were confirmed by DNA sequencing (Bio Matrix Research, Inc., Kashiwa, Chiba, Japan).

2.2. Cell culture

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 (10,000 U/ ml penicillin, 10,000 µg/ml streptomycin, and 25 µg/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% CO_2 at 37 °C. BAECs from passages 6–10 were used for the experiments.

2.3. Transfection

Prior to transfection, BAECs were seeded in a 35-mm glass-bottomed dish, and cultured to 70% confluence. BAECs adherent to the glass-bottomed dish were washed twice with opti-MEM (GIBCO™; Thermo Fisher Scientific). The DMEM was changed to 1 ml of opti-MEM, and the BAECs were cultured for 14–16 h. Two microliters of DNA (1 µg/µl) in 100 µl of opti-MEM was mixed with 4 µl of HilyMax (Dojindo Laboratories, Kumamoto, Japan). Then, this solution, including the construct DNA, was added to BAECs adherent to the glass-bottomed dish with 1 ml of opti-MEM, and PKCα–GFP was transfected into the BAECs. BAECs grown to confluence were used for experiments 2 days after transfection.

2.4. Immunoblotting

After 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-PKCα Clone M4 (EMD Millipore Corp., Billerica, MA, USA) and anti-GFP (Clontech Laboratories, Inc.) at a dilution of 1:10,000 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 1:10,000 for 1 h at room temperature. Finally, the membranes were probed with Pierce® High-Sensitivity Streptavidin-HRP (Thermo Fisher Scientific) at a dilution of 1:1000 for 1 h at room temperature.

2.5. 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-PKCα Clone M4 (Clontech Laboratories, Inc.) at a dilution of 1:200. The preparation was washed three times with PBS, and subsequently 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 $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM D-glucose, and 15 mM HEPES). Fluorescent images were captured by confocal microscopy (Digital Eclipse C1; Nikon Corp., Tokyo, Japan) using a 60× oil-immersion lens (S-Fluor, NA = 1.4; Nikon Corp.). Confocal images of GFP 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.

2.6. Confocal images of protein kinase Cα–green fluorescent protein translocation before and after ATP stimulation

Transfected BAECs were washed twice with HBS. Translocation of PKCα–GFP to the cell membrane was observed before and after the addition of 200 µM ATP (Sigma-Aldrich Corp., St. Louis, MO, USA). To measure the translocation of PKCα–GFP, we used confocal microscopy (Digital Eclipse C1) with a 60× oil-immersion lens (S-Fluor, NA = 1.4). Confocal images of PKCα–GFP 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.

2.7. Mechanical wounding

Mechanical wounding of BAECs was performed on a single cell using a fine glass micropipette. The tip of the pipette was pulled to be less than 4 µm in diameter (P-97; Sutter Instrument Company, Novato, CA, USA). The micropipette was fixed in a micromanipulator (MMO-203; Narishige Group, Tokyo, Japan). The tip of the micropipette was positioned over a single cell, and moved vertically to wound the cell mechanically. We determined whether the cell was wounded and dead by observing the decrease in the fluorescent intensity of the Ca^{2+} indicator Fura-2–acetoxymethyl ester (Fura-2–AM; Invitrogen, Carlsbad, CA, USA).

2.8. Simultaneous measurement of changes in intracellular Ca^{2+} concentration and protein kinase $C\alpha$–green fluorescent protein translocation

BAECs were cultured in a 35-mm glass-bottomed dish to 100% confluence for 48 h after transfection. Transfected BAECs were washed twice with HBS and then loaded with 5 mM Fura-2–AM in HBS for 45 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 40×

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 (C7501;Hamamatsu Photonics K.K.). The Fura-2–AM was excited with 380 nm, and the PKCα–GFP was excited with 488 nm. To simultaneously monitor the fluorescence of both intracellular Ca^{2+} and PKCα–GFP, the excitation wavelength was shifted between 380 and 488 nm at 148 ms intervals. The fluorescence
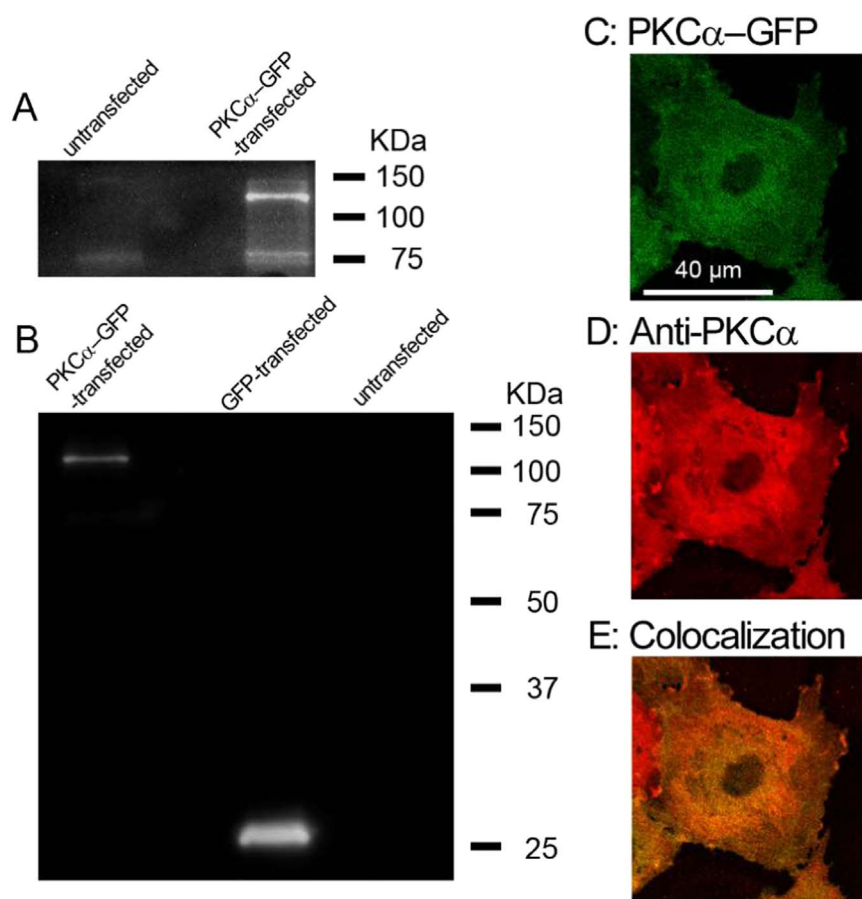
Fig. 1. Characterization of the protein kinase Cα–green fluorescent protein (PKCα–GFP) expressed in bovine aortic endothelial cells via western blot analysis, confocal microscopy, and ATP stimulation. (A) Immunoblots of cell lysates from untransfected and PKCα–GFP-transfected bovine aortic endothelial cells (BAECs) probed with an antibody against PKCα. (B) Immunoblots of cell lysates from PKCα–GFP-transfected, GFP-transfected, and untransfected BAECs probed with an antibody against GFP. (C) PKCα-GFP. (D) Anti-PKCα. (E) Colocalization. (F) Time-series image of PKCα–GFP localization before and after stimulation with ATP captured using confocal microscopy. Scale bar: 40 μm.
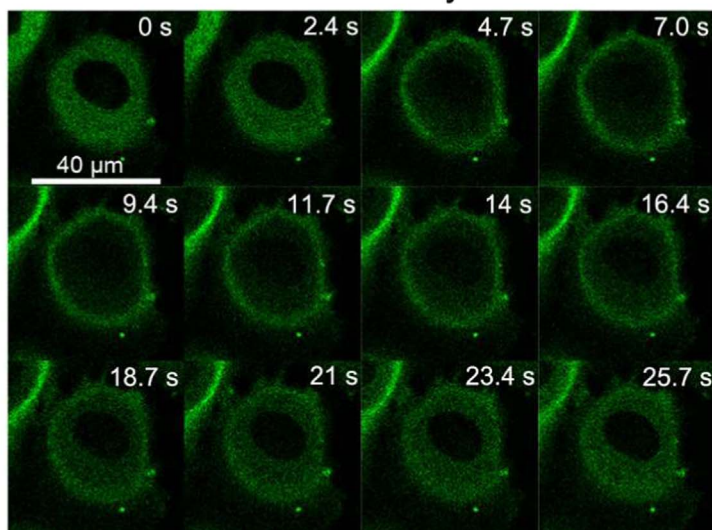
emission from the sample was amplified with an image intensifier (C8600-03; Hamamatsu Photonics K.K.), detected by a charge-coupled device camera (C6790; Hamamatsu Photonics K.K.), and recorded on a personal computer. The microscope stage was maintained at 37 °C by enclosure in thermal insulation material.

PKCα–GFP fluorescence and intracellular Ca^{2+} level increases were evaluated according to the relative fluorescent intensity in the region of interest within ECs. The relative intensity was defined as the fluorescent intensity at any time divided by the time-averaged intensity before stimulation during about 1.5 s (five images). The cell membrane region was defined as the area extending to a maximum depth of 5 μm from the edge of the cell in line with Almholt et al. [11].

2.9. Statistical analysis

Statistical analyses 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 are denoted by *N* and *n,* respectively.

3. Results

3.1. Protein kinase Cα–green fluorescent protein is expressed in bovine aortic endothelial cells

We first characterized the expression and localization of PKCα–GFP in BAECs by western blot analysis and compared the results with those for wild-type PKCα (Fig. 1A and B). PKCα was detected at approximately 80 kDa. Anti-PKCα antibody yielded a band at approximately 110 kDa in PKCα–GFP-transfected cells. GFP was detected at approximately 25 kDa, and PKCα–GFP was detected at 110 kDa, similar to anti-PKCα.

Next, we observed BAECs transfected with PKCα–GFP using confocal microscopy (Fig. 1C–E). PKCα–GFP was distributed homogenously based on the direct observation of GFP fluorescence. PKCα was distributed in the cytosol based on the observation of anti-PKCα immunofluorescence. The colocalization of PKCα–GFP fluorescence and anti-PKCα immunofluorescence demonstrated that the fusion protein was localized in the same area as PKCα.

We also examined PKCα–GFP translocation after the addition of ATP, which is also known to activate PKCα, using confocal microscopy (Fig. 1F). Before the addition of ATP (at 0 s), PKCα–GFP was distributed homogeneously throughout the cytoplasm, and absent in the nucleus. Stimulation with 200 μM ATP caused rapid and transient translocation of PKCα–GFP to all parts of the plasma membrane and its depletion in the cytoplasm. After 18.7 s, PKCα–GFP gradually returned to a homogeneous distribution throughout the cytoplasm, and was excluded from the nucleus.

3.2. Simultaneous monitoring of intracellular Ca^{2+} dynamics and Protein kinase Cα–green fluorescent protein translocation after the addition of ATP

Fig. 2 shows the simultaneous images of PKCα–GFP and intracellular Ca^{2+} levels after the addition of 200 μM ATP. As shown in Fig. 2A, ATP caused a decrease in the fluorescence intensity of Fura-2–AM in all cells, indicating an increase in intracellular Ca^{2+}. Before the addition of ATP (at 0 s), PKCα–GFP was distributed in the cytosol, especially around the nucleus. After the addition of ATP, PKCα–GFP translocated to the cell membrane (Fig. 2B). In contrast to the fluorescent images acquired by confocal microscopy in Fig. 1F, the membrane was not clearly visible. In this experiment, confocal microscopy was not used due to the lack of time resolution. To demonstrate PKCα translocation, the image before stimulation (at 0 s in Fig. 2B) was subtracted from each image (Fig. 2B). As a result, the fluorescent intensity was distributed at the edge of the cell (Fig. 2C), suggesting that PKCα–GFP translocated to the cell membrane after ATP stimulation.

Fig. 2D shows the simultaneous analysis of intracellular Ca^{2+} levels and PKCα–GFP localization in the cell membrane region following extracellular ATP stimulation. Here, the cell membrane region was assumed to be the area extending to a maximum depth of 5 μm from the edge of the cell [11]. ATP stimulation led to a statistically significant decrease in the relative fluorescent intensity of Fura-2–AM, equivalent
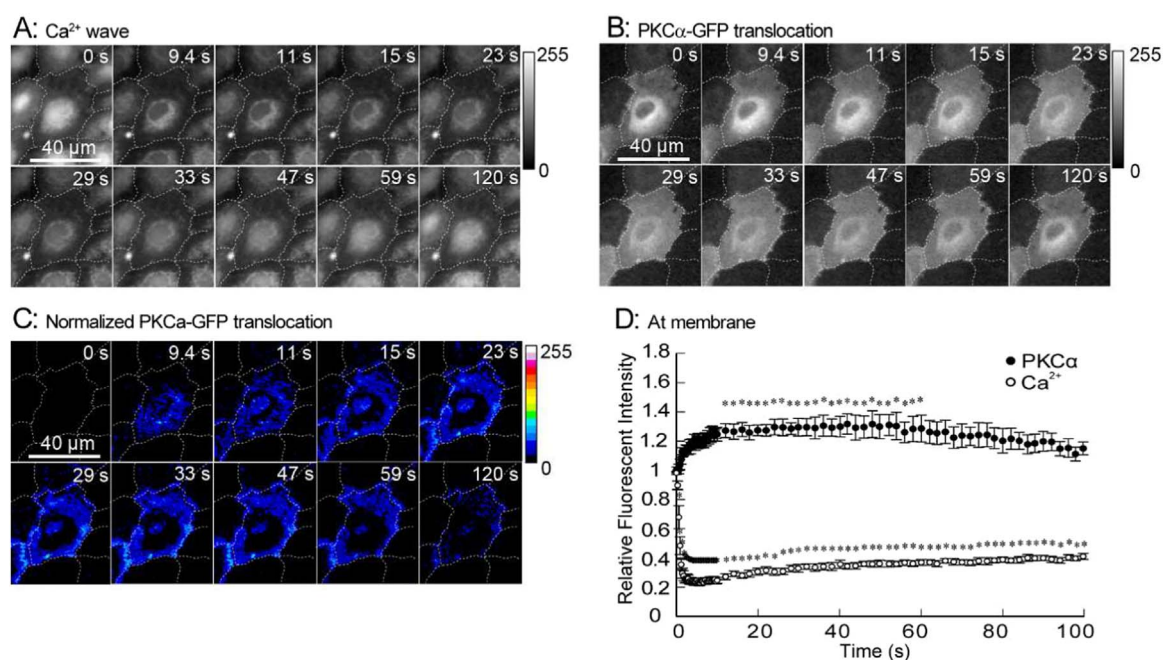
Fig. 2. Time-series analysis of the ATP-induced increase in intracellular Ca^{2+} and protein kinase Cα–green fluorescent protein (PKCα–GFP) translocation. (A) Increase in intracellular Ca^{2+}, (B) PKCα–GFP translocation, and (C) normalized PKCα–GFP translocation in response to 200 μM ATP added immediately after *t* = 0. Normalized PKCα–GFP translocation is time-series images after subtracting the images at *t* = 0 from every time-series image in (B). Scale bar: 40 μm. (D) Time-series analysis of the relative fluorescent intensity of intracellular Ca^{2+} and protein kinase Cα–green fluorescent protein in the cell membrane. *N* = 9, *n* = 36. Relative fluorescent intensity is presented as the mean ± standard error of the mean. *P < 0.05 vs. 0 s.

to a statistically significant increase in intracellular Ca^{2+} concentration, within 1 s. Additionally, an intracellular Ca^{2+} peak was observed 5.9 s after ATP stimulation. ATP induced a statistically significant increase in the relative fluorescent intensity of PKCα–GFP after 2 s and a peak 8.5 s after ATP stimulation. This increase in fluorescent intensity persisted until 30 s.

3.3. Simultaneous monitoring of intracellular Ca^{2+} levels and protein kinase Cα translocation before migration

Fig. 3A–C are representative fluorescent images of the changes in intracellular Ca^{2+} levels and PKCα–GFP translocation in the cells which were adjacent to the mechanically wounded cell. A video of PKCα–GFP translocation is shown in Movie S1. The mechanically wounded point is marked by "×" at 0 s in Fig. 3A–C and D. The fluorescence of Ca^{2+} did not returned to an entire level after mechanical stimulation (Fig. 3A), indicating that this wounded cell was dead. In the neighboring cells surrounding the wounded cell (Fig. 3D), the fluorescent intensities decreased immediately after the mechanical wounding, indicating the propagation of an intracellular Ca^{2+} wave before migration (Fig. 3A). As shown in Fig. 3B and C and Movie S1, PKCα–GFP was distributed homogeneously in the cytosol before mechanical stimulation. After mechanical wounding, PKCα–GFP had translocated to the cell

membrane at about 10 s (9.7 s in Fig. 3B and C), similar to that after ATP stimulation (shown in Fig. 2). In contrast to ATP stimulation, after 10 s, there was a time-dependent increase in PKCα–GFP fluorescence at the periphery adjacent to the wounded cell (indicated by arrows in Fig. 3B and C), and this accumulation was sustained for about 60 s. After 90 s, PKCα–GFP returned to the homogeneous distribution that was evident before stimulation.

We further analyzed the fluorescent intensity of two segments of the neighboring cells, near and far, as defined by their distance relative to the mechanically wounded cell and based on the definition that the cell membrane extended to a maximum depth of 5 μm from the edge of the cell (Fig. 3D). Fig. 3E and F illustrate the changes in the ratios of the mean fluorescent intensities of intracellular Ca^{2+} and PKCα–GFP in neighboring cells of the wounded cell. The relative fluorescent intensity of intracellular Ca^{2+} decreased after mechanical wounding, and showed a statistically significant decrease at 1.2 and 2.1 s in the near and far segments, respectively. The relative fluorescent intensity of PKCα–GFP increased after mechanical wounding, and showed a statistically significant increase at 4.4 and 5.6 s in the near and far segments, respectively. However, after about 10 s, different phenomena were evident in the near and far segments. In the far segment, the relative fluorescent intensity of PKCα–GFP decreased and returned to the level evident before stimulation, whereas in the near segment, the
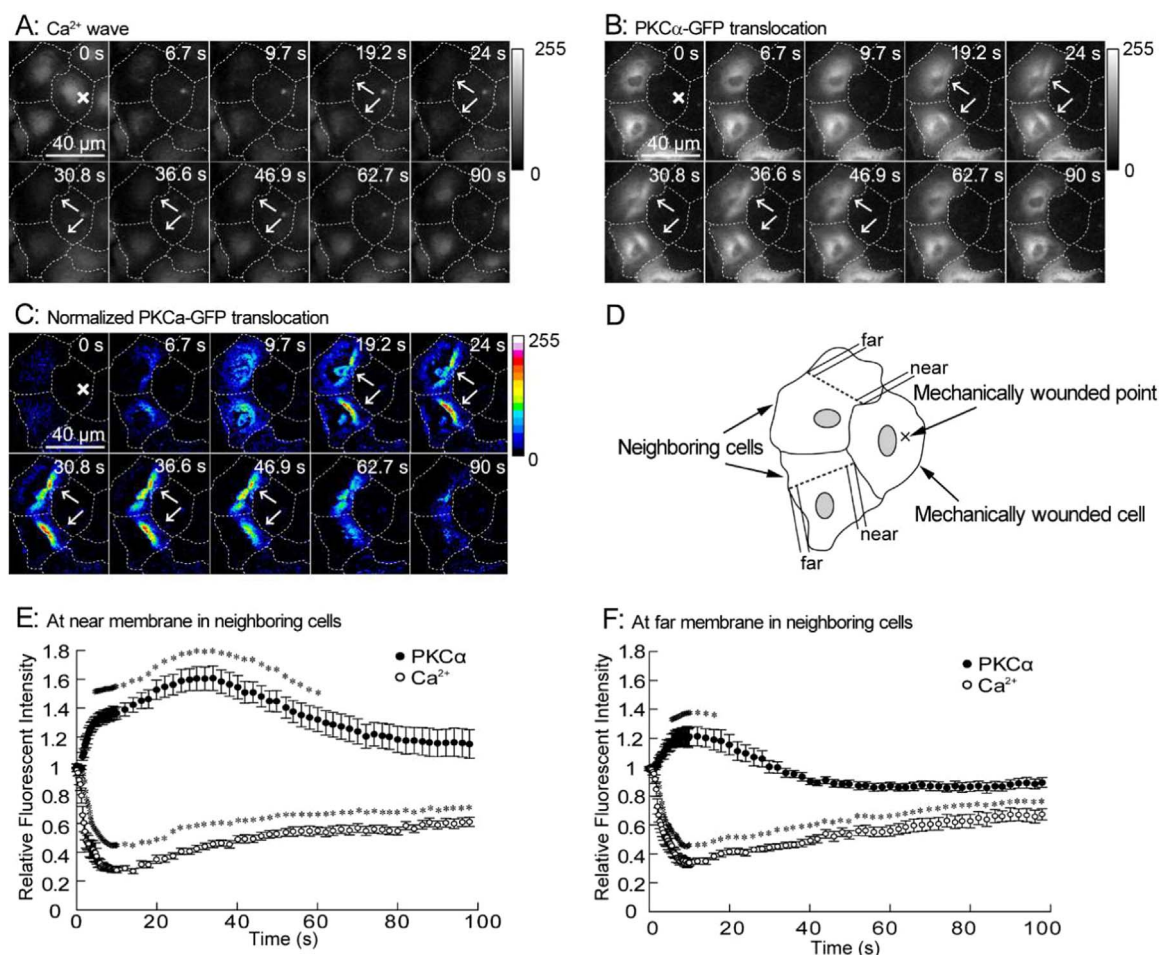
Fig. 3. Time-series analysis of the increase in intracellular Ca^{2+} and protein kinase Cα–green fluorescent protein (PKCα–GFP) translocation before cell migration. (A) Increase in intracellular Ca^{2+}, (B) PKCα–GFP translocation (a video image of PKCα translocation is shown in Movie S1), and (C) normalized PKCα–GFP translocation after mechanical wounding. Normalized PKCα–GFP translocation was obtained by subtracting the images at $t = 0$ from every time-series image in (B), and the intensity of PKCα–GFP is shown using a pseudo color scale. Scale bar: 40 μm. "×" indicates the point wounded by a micropipette. (D) The definition of far and near segments in neighboring cells of the mechanically wounded cell. Time-series analysis of intracellular Ca^{2+} and PKCα–GFP in the near (E) and far (F) plasma membrane segments, defined by the distance relative to the mechanically wounded cell. $N = 10$, $n = 14$. Relative fluorescent intensity is presented as the mean ± standard error of the mean. *$P < 0.05$ vs. 0 s.
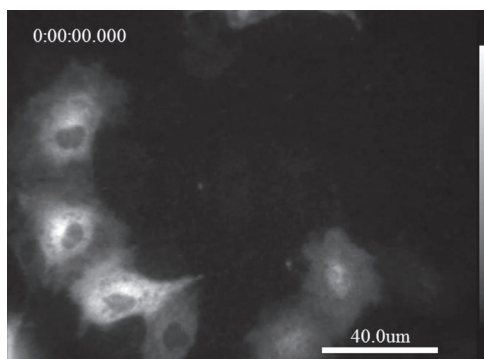
Video S1. PKCα–GFP translocation in the BAECs after mechanical wounding. Supplementary material related to this article can be found online at http://dx.doi.org/%2010.1016/j.bbrep.2017.08.003.

relative fluorescent intensity further increased after 10 s and showed a second peak at about 34 s; at about 90 s, relative fluorescent intensity returned to the level evident before stimulation.

4. Discussion

In this study, we investigated PKCα translocation in the BAECs that are adjacent to the mechanically wounded cell (Fig. 3D), particularly focusing on the period before cell migration. We have shown for the first time that the mechanical wounding of BAECs causes biphasic and directed translocation of PKCα–GFP in BAECs adjacent to mechanically wounded cells before the migration to the denuded area. In the first phase of translocation, PKCα translocated to the cell membrane similarly to what occurs after chemical stimulation. However, the second phase of translocation did not recapitulate the results previously shown

after chemical stimulation, but PKCα was accumulated at the edges of cells adjacent to the mechanically wounded cells.

To track the translocation of PKCα, we used PKCα–GFP. This fusion protein was initially distributed throughout the cytosol of BAECs, but translocated to almost all parts of the plasma membrane after the addition of ATP. PKCα is also known to translocate to the membrane following ATP stimulation. Marín-Vicente et al. [6] investigated intracellular Ca^{2+} dynamics and PKCα translocation in neural growth factor-differentiated pheochromocytoma cells subjected to extracellular stimulation with ATP. They showed that an intracellular Ca^{2+} peak occurs 10 s after ATP stimulation; they also showed that PKCα translocation to the plasma membrane results in maximal cell-membrane localization 20 s after stimulation with 100 μM ATP. In our experiment, an intracellular Ca^{2+} peak occurred 5.9 s after ATP stimulation; PKCα translocation to the plasma membrane resulted in maximal cell-membrane localization 8.5 s after ATP stimulation. Compared with previous results, although there was a difference in the time of peak PKCα translocation, our fusion protein displayed the properties of the intact protein.

Furthermore, in our experiments, PKCα–GFP affected by an ATP stimulus translocated to almost all parts of the plasma membrane. In contrast, when the single cell was wounded mechanically and dead, PKCα–GFP in the neighboring cells translocated to almost all parts of the plasma membrane of cells and then accumulated at the cell membrane adjacent to the wounded cell before migration. This biphasic and directed translocation of PKCα–GFP of the neighboring cells shows a pattern different from the translocation induced by chemical stimulation. It remains unclear why PKCα accumulated at the edge of cells adjacent to mechanically wounded cells in this study. There are two possible explanations. First, chemical substances may be transported from mechanically wounded cells to neighboring cells via gap-junctional transport and paracrine signaling events such as ATP release.
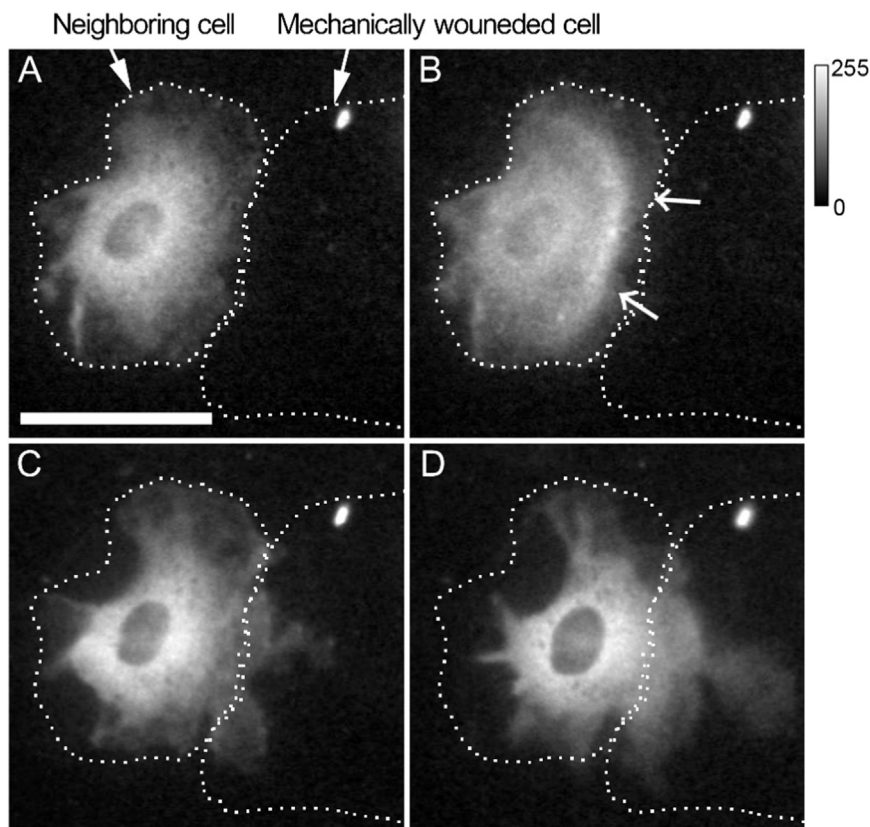
Fig. 4. Relationship between cell migration and the directed protein kinase Cα–green fluorescent protein (PKCα–GFP) translocation in the neighboring cell of a mechanically wounded cell. (A) Before the mechanical wounding, (B) the directed PKCα–GFP translocation, (C) 1 h after wounding, and (D) 3 h after wounding. The initial geometries of the mechanically wounded cell and its neighboring cell, respectively, are shown by dotted lines. PKCα–GFP was not expressed in the mechanically wounded cell in (A). Arrows in (B) indicate the directed PKCα–GFP translocation to the wounded cell. Scale bar is 40 μm.

Mechanically stimulated cells are known to release chemical substances such as ATP [12]. Indeed, some chemical substances are known to induce intracellular Ca^{2+} waves in neighboring cells [12–14]. Gomes et al. [15] investigated the contribution of gap junctions to intercellular communication in cultured bovine corneal ECs. They showed that Gap 27, a connexin-mimetic peptide that blocks gap junctions, reduces the Ca^{2+} wave propagation induced by mechanical stimulation with a glass micropipette. Furthermore, they showed that both gap junctions and paracrine signaling events interact. Extracellular chemical substances use paracrine signaling, intercellular chemical substances use gap junctions, and both may induce the localized accumulation of PKCα. Further, there may be a direct mechanical effect on neighboring cells due to the dissipation of mechanical stress between mechanically wounded cells and neighboring ones. Lansman et al. [16] investigated stretch-activated (SA) ion channels in porcine pulmonary ECs using the patch-clamp method. They reported that the channel was permeable to Ca^{2+}, and its opening frequency increased when the membrane was stretched by applying suction through the patch electrode. Naruse and Sokabe [17] investigated SA ion channels related to intracellular Ca^{2+} mobilization in human umbilical vein endothelial cells (HUVECs) affected by mechanical stretching. They showed that stretching the cell membrane increased intracellular Ca^{2+} levels in HUVECs, and suggested that this increase was due to SA channels. In our experiments, after mechanical wounding, the conformation of cell–cell junctions was destroyed; therefore, suggesting an alteration in mechanical stress. Mechanical SA channels on the cell membrane of cells adjacent to mechanically wounded cells may be activated locally. This localized activation of SA channels may, in turn, activate PKCα. Further studies are needed to reveal this mechanotransduction signaling, such as using inhibitors/antagonists and with different cell–cell interactions.

In this study, we investigated PKCα kinetics before cell migration and not cell migration to the denuded area. Thus, we focused on the cells that are adjacent to the mechanically wounded cell. A scratched wound area was implemented across the cell monolayer after the cells were cultured to 100% confluence to investigate cell migration [18–20]. A previous study reported that a Ca^{2+} wave is induced in the cells adjacent to the wound area immediately after mechanical wounding, and their subsequent motility is initiated and regulated by this Ca^{2+} wave [5]. In this study, the single cell was wounded and killed microscopically, resulting in not only a Ca^{2+} wave but also PKCα translocation being induced in neighboring cells. Finally, we examined the relationship between the directed PKCα–GFP translocation and cell migration, and found that PKCα translocated toward the wounded cell, followed by cell migration to the denuded area (Fig. 4). Both Ca^{2+} wave and PKCα translocation are involved in the signal pathway in various ECs, even though the endothelial functions may be heterogeneous. Indeed, the regulation of the cellular motility by Ca^{2+} wave was reported in calf pulmonary artery ECs [5], and PKCα translocation was also observed in various ECs, such as human dermal microvascular ECs [2], human umbilical vein ECs [4], and porcine aortic ECs [21]. Although the mechanism underlying the biphasic and directed distribution of PKCα during a Ca^{2+} wave remains unclear, our results based on BAECs suggest that the directed translocation of PKCα before migration may regulate the initiation of subsequent directed motility.

Acknowledgments

Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.08.003.

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