Mechanical stiffness softening and cell adhesion are coordinately regulated by ERM dephosphorylation in KG-1 cells

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Author contributions
Teru Matsumoto and Yoshihito Nakahashi perfomed AFM analysis. Kouichi Tachibana performed immunoblotting. Takanori Kihara performed cell adhesion experiments and is responsible for the whole of this study. All authors have read and agreed to the final

version of the manuscript.

Abstract

Mechanical stiffness is closely related to cell adhesion and rounding in some cells. In leukocytes, dephosphorylation of ezrin/radixin/moesin (ERM) proteins is linked to cell adhesion events. To elucidate the relationship between surface stiffness, cell adhesion, and ERM dephosphorylation in leukocytes, we examined the relationship in the myelogenous leukemia line, KG-1, by treatment with modulation drugs. KG-1 cells have ring-shaped cortical actin with microvilli as the only F-actin cytoskeleton, and the actin structure constructs the mechanical stiffness of the cells. Phorbol 12-myristate 13-acetate and staurosporine, which induced cell adhesion to fibronectin surface and ERM dephosphorylation, caused a decrease in surface stiffness in KG-1 cells. Calyculin A, which inhibited ERM dephosphorylation and had no effect on cell adhesion, did not affect surface stiffness. To clarify whether decreasing cell surface stiffness and inducing cell adhesion are equivalent, we examined KG-1 cell adhesion by treatment with actin-attenuated cell softening reagents. Cytochalasin D clearly diminished cell adhesion, and high concentrations of Y27632 slightly induced cell adhesion. Only Y27632 slightly decreased ERM phosphorylation in KG-1 cells. Thus, decreasing cell surface stiffness and inducing cell adhesion are not equivalent, but these phenomena are coordinately regulated by ERM dephosphorylation in KG-1 cells.

Keywords

Leukocytes; ERM; Mechanical stiffness; Cell adhesion; Cortical actin

Introduction

Mechanical properties and forces drive the modulation of cell shape, motility, and tissue morphogenesis [1]. For example, changes in the mechanical properties of cells drive mitotic cell rounding [2-4], and changes in the stiffness of the retinal epithelium and mechanical forces are important for optic-cup morphogenesis [5, 6]. The mechanical properties, especially cell surface stiffness, are largely attributable to the actin cytoskeleton [7-10]. Thus, surface stiffness changes during cell events through reorganization of the actin cytoskeleton.

HEK293 cells, which exhibit immature cytoskeletal actin filaments, show very low stiffness in the substrate adhesion state; however, by cell detachment, the stiffness clearly increases [11]. Moreover, in the process of cell detachment, the cell shape becomes spherical and clear cortical actin is formed at the plasma membrane, which provides mechanical strength [11]. How do HEK293 cells form mature cortical actin by stimulating cell detachment? We hypothesized that ezrin/radixin/moesin (ERM) proteins, which are cross-linkers between transmembrane proteins and F-actin in the cortical region, regulate cortical actin maturation [11]. The ERM proteins of adhered HEK293T cells are almost dephosphorylated, but it has been reported that they are phosphorylated by cell detachment from the culture surface [12]. Furthermore, phospho-mimetic moesin-expressing HEK293T cells showed augmentation of cell surface stiffness and cell rounding [13]. Thus, cell surface mechanical stiffness is closely related to cell adhesion and rounding, as well as ERM phosphorylation in HEK293 cells.

Recently, it was reported that the phosphorylation level of ERMs is important for cell adhesion in leukocytes [14]. Leukocytes are non-adherent and spherically shaped cells, and they usually phosphorylate ERMs at the plasma membrane [15, 13, 16]. ERMs are dephosphorylated by stimulation with chemokines (stromal derived factor 1 (SDF-1) or secondary lymphoid tissue cytokine) in leukocytes [15, 14]. By inhibiting the dephosphorylation of ERMs with the serine/threonine phosphatase inhibitor calyculin A, the polarization and stimulation of T lymphocytes is inhibited [15]. In KG-1 cells, which are non-adherent cells derived from acute myelogenous leukemia, the strong kinase inhibitors staurosporine and phorbol ester (phorbol 12-myristate 13-acetate, PMA) induce dephosphorylation of ERMs and cell adhesion to the substrate, a characteristic of

stimulated leukocytes [13, 14]. The PMA-induced cell responses, which include ERM dephosphorylation and induction of cell adhesion, were completely inhibited by treatment with calyculin A or transfection with phospho-mimetic moesin expression vector [14]. Thus, the phosphorylation level of ERM is an essential regulator of leukocyte adhesion and polarization.

The behaviors of cell adhesion and ERM phosphorylation alterations in leukocytes and HEK293T cells are correspondent; ERMs of the non-adherent floating states of these cells are phosphorylated and those of the surface adherent states are dephosphorylated [12-14]. If so, it is thought that the cortical actin of leukocytes will be changed by the phosphorylation level of ERMs. In fact, the surface stiffnesses of KG-1 cells and murine T-cell hybridoma 3A9 cells were drastically reduced by PMA treatment [14, 17]. It remains to be elucidated whether the maturation of cortical actin is related to cell adhesion and stimulation of leukocytes, and whether ERMs act as key regulators of this phenomenon. Here, we examined the relationship between mechanical stiffness and cell adhesion of KG-1 cells by treatment with modulation drugs for altering ERM phosphorylation levels.

Materials and Methods

Materials

The cone probe (BL-AC-40TS-C2; spring constant: approximately 0.05 N/m) was purchased from Olympus (Tokyo, Japan). The pyramidal probe (SN-AF03; spring constant: approximately 0.08 N/m) was purchased from Hitachi High-Tech (Tokyo, Japan). KG-1 cells were obtained from RIKEN Bio Resource Center (Ibaraki, Japan). The cell anchoring molecule, SUNBRIGHT OE-020CS, was purchased from NOF Corporation (Tokyo, Japan). Fibronectin from bovine serum was purchased from Life Laboratory Company (Yamagata, Japan). The cell culturing micro-flow channel, μ-slide VI 0.4, was purchased from ibidi (Grafelfing, Germany). Anti-phosphorylated ERM (p-ERM) antibody was purchased from Cell Signaling Technologies (Danvers, MA). Anti-β-actin antibody (AC-74) was purchased from Sigma-Aldrich (St. Louis, MO). Other reagents were purchased from Sigma-Aldrich, Wako Pure Chemical Industries Ltd.

(Osaka, Japan), or Life Technologies Japan Ltd. (Tokyo, Japan).

Preparation of cell anchoring dishes

Cell anchoring dishes were prepared as previously described [18, 19]. The cell anchoring molecule, SUNBRIGHT OE-020CS, contains an oleyl group at one end that anchors the suspended cells [20]. Briefly, culture dishes were coated with 2% BSA and then treated with 1 mM SUNBRIGHT OE-020CS. The cell-anchoring dishes were then washed and dried.

Cell culture and drug treatment

KG-1 cells were maintained in RPMI medium containing 10% FBS and antibiotics (penicillin and streptomycin). For AFM measurement, KG-1 cells were plated onto the cell anchoring dish for 1.5 h in serum-free culture medium, and then washed with complete culture medium to remove unattached cells. The cells were then treated with staurosporine, PMA, calyculin A, cytochalasin D, or Y27632 for 1.5 h.

Cell adhesion assay

Adhesion of KG-1 cells to fibronectin-coated surfaces was performed in a micro-flow channel, ibidi μ-slides. The channels were coated with 50 μg/mL fibronectin for 60 min. After washing with culture medium, 50 μL of KG-1 cell suspension (1.6×10^6 cells/mL) was plated in each channel, and the cells were cultured with or without drugs for 3 h in a channel. Then, unattached cells were removed by washing with culture media, and phase contrast microscopic images of attached cells were obtained. The number of attached cells in each image was counted using ImageJ software (NIH, Bethesda, MD).

Fluorescent image of the actin cytoskeleton

To visualize actin cytoskeletal microstructures, KG-1 cells were anchored on cell-anchoring glass bottom dishes. The anchored cells were treated with drugs, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained with rhodamine phalloidin. The specimens were observed by confocal laser scanning microscopy (CLSM) (Nikon C2; Nikon, Tokyo, Japan).

AFM measurements

The cell anchoring dish-fixed KG-1 cells in complete medium were manipulated by AFM (Nanowizard III; JPK Instruments AG, Berlin, Germany) using an optical microscope (IX-71; Olympus) at room temperature. The AFM probe was indented at the apex of the cell with a loading force of up to 0.5 nN and velocity of 1 μm/s. Young's modulus was determined as described previously [19, 21, 11]. More than 20 cells were used per experiment, and 25 points were indented on the 1 μm × 1 μm area of each cell apex; thus, we analyzed more than 500 force curves in each condition. The logarithmic values of Young's modulus were used for statistical analysis.

Immunoblotting

KG-1 cells were incubated with the reagents for 10 min in culture media before harvesting. Cellular lysates were prepared in Tris-buffered saline containing 1% CHAPS, 10 mM NaF, 5mM $NaPP_i$, 1 mM Na_3VO_4, 0.5 mM PMSF, and protease inhibitor cocktail. Lysates were then analyzed by SDS-PAGE and immunoblotting using a previously described method [13]. Images were captured using LAS4000mini (Life Technologies) and analyzed with ImageJ software. The relative ratio of anti-phospho-ERM antibody was normalized by immunoblotting with an anti-β-actin antibody.

Statistical analysis

The obtained values were compared using one-way analysis of variance and Dunnett's pairwise comparison test.

Results

PMA and staurosporine induce KG-1 cell adhesion to fibronectin substrate

First, we checked the stimulatory effect of PMA and staurosporine on the adhesion of KG-1 cells to plasma fibronectin substrates. KG-1 cells are non-adherent myeloblast cells derived from the human bone marrow, and it has been reported that PMA and staurosporine induce KG-1 cell adhesion to fibronectin CS-1 peptide, a ligand for α4β1

integrin, by dephosphorylation of ERMs [13, 14]. In this experiment, we cultured KG-1 cells in a fibronectin-coated microflow channel, to wash out weakly attached cells by flow. In the non-treated conditions, approximately 28 cells/mm^2 were adhered to the fibronectin surface (Fig. 1). PMA and staurosporine clearly induced adhesion of KG-1 cells to fibronectin surfaces; approximately 200-300 cells/mm^2, which were approximately 30–50% of the seeded cells, were adhered (Fig. 1). On the other hand, the serine/threonine protein phosphatase inhibitor calyculin A, which inhibits stimulus-inducing dephosphorylation of ERMs [14], had no effect on KG-1 cell adhesion to the fibronectin surface (Fig. 1). These results are in agreement with those of previous reports [13, 14], whereby PMA and staurosporine stimulated KG-1 cell adhesion to the fibronectin surface.

Stiffness changes in stimulated KG-1 cells

Before measuring the surface stiffness of KG-1 cells, we observed the actin cytoskeleton of KG-1 cells using CLSM (Fig. 2A). Only ring-shaped and microvillus F-actin structures were observed at the plasma membrane (Fig. 2A). Upon treatment with actin-depolymerizing agent cytochalasin D, F-actin granules were observed, the ring-shaped cortical actin was faded, and the microvilli vanished (Fig. 2A).

We then determined the surface stiffness of KG-1 cells with stimuli using the AFM indentation method. The distribution of Young's modulus of KG-1 cells is shown in Fig. 2B. The median value of Young's modulus of regular KG-1 cells was approximately 1.9 kPa (Fig. 2B). When the cells were treated with cytochalasin D, Young's modulus of KG-1 cells dropped to 0.21 kPa (Fig. 2B). Thus, the surface stiffness of KG-1 cells was largely derived from cortical actin. Furthermore, the Rho kinase (ROCK) inhibitor Y27632, which decreases the surface stiffness of many cell types [22, 11], also decreased the surface stiffness of KG-1 cells (Fig. 2B). When KG-1 cells were treated with PMA or staurosporine, Young's modulus of the cell surface decreased about to 0.75 and 0.36 kPa, respectively (Fig. 2B). In contrast, the Young's modulus of KG-1 cells treated with calyculin A did not result in any change (Fig. 2B). Overall, PMA and staurosporine, which are the stimuli of ERM dephosphorylation, decreased the Young's modulus of KG-1 cells, but calyculin A, which inhibits ERM dephosphorylation, did not

affect the surface stiffness. Thus, the surface stiffness of KG-1 cells decreased after treatment with the stimuli, which induced the dephosphorylation of ERM proteins and cell adhesion.

Relation between cell softening and substrate adhesion in KG-1 cells

PMA and staurosporine, which induced KG-1 cell adhesion, decreased the surface stiffness. This raised the question of whether decreasing cell surface stiffness and inducing cell adhesion are equivalent in KG-1 cells and in leukocytes in general. 3D computational simulation indicates softer leukocytes can deform easily and increase contact area under flow shear [23]. If so, softened KG-1 cells adhere to the fibronectin substrate. We then examined KG-1 cell adhesion to fibronectin substrate by treatment with the actin attenuation cell softening reagents cytochalasin D and Y27632. When KG-1 cells were treated with cytochalasin D, the number of adhered cells was clearly diminished (Fig. 3). On the other hand, when KG-1 cells were treated with Y27632, the adhered cell counts showed an unusual trend; they slightly decreased in the presence of low concentrations of Y27632 but increased in presence of high concentrations of Y27632 (Fig. 3) Thus, adhesion to the fibronectin surface and cell surface softening were not equivalent, and KG-1 cells showed both phenomena only when ERM proteins were dephosphorylated. The question remains as to why Y27632 had an unusual effect on KG-1 cell adhesion. Notably, high concentrations of Y27632 reduced the Young's modulus of KG-1 cells as well as low concentrations did (Supplementary Figure S1).

KG-1 cell adhesion requires ERM dephosphorylation

We then examined the phosphorylation level of ERMs following treatment of KG-1 cells with actin attenuation reagents. Phosphorylated ERM was observed in non-treated KG-1 cells, which was almost abrogated by treatment with PMA (Fig. 4). Phosphorylated ERM levels in the KG-1 cells treated with Y27632 slightly decreased, and cells treated with cytochalasin D was almost unchanged (Fig. 4). Thus, Y27632 attenuated actin filaments and induced ERM dephosphorylation. That is, the reagents decrease phosphorylated ERM level-induced cell adhesion, and softened the surface stiffness in KG-1 cells; however, the reagent without changes to phosphorylated ERM levels did not induce the

cell adhesion. Therefore, ERMs are an important regulator of changes in surface stiffness and cell adhesion in KG-1 cells. ERMs maintain cortical actin structure and cell surface stiffness and inhibit cell adhesion. Dephosphorylation of ERMs destabilizes cortical actin, decreases cell surface stiffness, and increases cell adhesion.

Discussion

In this study, we demonstrated that ERM dephosphorylation and the induction of cell adhesion reduced cell surface stiffness in floating myeloblast KG-1 cells. On the other hand, cell softening reagents without ERM-dephosphorylation effects, such as cytochalasin D, did not induce KG-1 cell adhesion. This is the first report of the relationship between mechanical stiffness, cell adhesion, and the ERM function of leukocytes.

ERM proteins link the cortical actin to transmembrane proteins and regulate cortex tension and stiffness [13, 24, 16]. To change the surface cortical actin architecture, it is necessary to inactivate ERM proteins and dissociate the actin from the membrane in leukocytes [25, 16]. ERM inactivation decreases surface stiffness in Drosophila S2R+ embryonic cells [3]. Our results also showed that PMA or staurosporine treatment, which induces the dephosphorylation of ERM proteins, reduced the surface stiffness of KG-1 cells (Fig. 2). Thus, ERM is a factor that regulates the surface stiffness of leukocytes.

On the other hand, cell adhesion and surface stiffness are different cell phenomena, in which cell adhesion is regulated by adhesion molecules such as integrin, and the surface stiffness is organized by cortical actin in KG-1 cells. Recently, we suggested that for leukocytes to deform their spherical shape and to generate flat attachment sites, they will need to reduce cell surface rigidity [14]. Furthermore, a 3D computational simulation indicated that softer leukocytes can deform easily and increase their contact area under flow shear [23]. In other words, soft leukocytes are more advantageous for adhesion because they can expand the contact area. In fact, in 3A9 hybridoma cells softened by PMA treatment, the force required for detachment from an ICAM-1 immobilized surface increases without changing the mechanical strength of the individual molecular interaction [17]. In this study, PMA and staurosporine, which

induced cell adhesion, softened the cells (Figs. 1 and 2). However, the actin-depolymerized, softened KG-1 cells did not adhere to the fibronectin surface (Fig. 3). Cell adhesion requires the activation of adhesion molecules or the inactivation of inhibitory molecules considering the cell adhesion from the perspective of surface molecules in leukocytes. In a previous study, no obvious alteration in the surface expression of adhesion molecule α4β1 integrin or adhesion inhibitory molecule CD34 sialomucin was observed with PMA treatment; however, in PMA-treated KG-1 cells, only CD34 was excluded from surface attachment sites [14]. ERM dephosphorylation enables the mobilization and exclusion of cell adhesion inhibitory sialomucins from attachment sites [14]. Furthermore, in monocyte adhesion, actin polymerization stabilizes α4β1 integrin-mediated adhesion [26]. Therefore, it is likely that cytochalasin D-treated softened KG-1 cells did not show any cell adhesion to fibronectin, because their ERM phosphorylation level was unchanged and the integrin-linked cortical actin was depolymerized (Figs. 2, 3, and 4). That is, the process of adhesion of leukocytes involves not only molecular mechanisms but also mechanical mechanisms such as the ease of increasing the contact area, both of which are controlled by ERM.

Whereas Y27632 softened KG-1 cells, it did not induce cell adhesion as did PMA or staurosporine, nor did it completely inhibit cell adhesion as did cytochalasin D (Figs. 3 and 4). A previous study has shown that Y27632 decreases augmented ERM phosphorylation in systemic lupus erythematosus T lymphocytes [27]. Furthermore, Y27632 enhances neutrophil adhesion [28, 29]. Our results showed that Y27632 slightly induced ERM dephosphorylation (Fig. 4). This suggests that ERM phosphorylation is weakly induced via ROCK in KG-1 cells. On the other hand, the inhibition of ROCK by Y27632 suppresses actin filament bundling through the inhibition of actomyosin contraction and depolymerizes actin filaments through inhibition of the LIMK-cofilin pathway, resulting in attenuated actin fibers [30-32]. This means that Y27632 acts negatively on the mechanical stabilization of integrin-mediated adhesion. That is, Y27632 potentially induces cell adhesion by softening cells and weakening ERM phosphorylation and inhibits cell adhesion through an insufficient actin structure. This contradictory phenomenon may have led to the unusual behavior of KG-1 cell adhesion (Fig. 3).

In this study, calyculin A showed no effect on the surface stiffness and adhesion ability of KG-1 cells (Figs. 1 and 2). Treatment with calyculin A increases the level of phosphorylated ERM in human peripheral blood T lymphocytes [14, 15]. Further, calyculin A activates actomyosin formation and enhances actin polymerization [33], and increases surface stiffness of cancer cells and adherent HEK293 cells [22, 11]. Thus, it seemed likely that calyculin A would also increase the stiffness of KG-1 cells. By contrast, calyculin A does not induce a further increase in the surface stiffness of normal stromal cells and suspended HEK293 cells, which originally show high surface stiffness [22, 11]. In this study, the surface stiffness of KG-1 cells was sufficiently high and ERMs were well phosphorylated (Figs. 2 and 4). Thus, it is assumed that the surface stiffness of KG-1 cells showed almost no change with calyculin A because there was little scope for further increasing their stiffness.

Finally, we would like to discuss whether the findings in this study can be extended to other cells. In this study, we used KG-1 myelogenous leukemia cells. The ERM dephosphorylation effects of PMA and staurosporine and the relationship between the ERM dephosphorylation and cell adhesion are well studied in KG-1 cells [13, 14]. The surface microvilli and cortical actin structure are common features in leukocytes, and the mechanisms regulating ERM dephosphorylation and cell adhesion in KG-1 cells are the same as those in normal peripheral T lymphocytes [14]. Therefore, we believe that our finding, that cell adhesion and surface stiffness are closely related and regulated by ERM is not limited to KG-1 cells. In fact, PMA treatment drastically reduced the surface stiffnesses of murine T-cell hybridoma 3A9 cells [17], and we also observed that PMA and staurosporine decreased cell surface stiffness of human T cell line Jurkat cells (data not shown). However, these results alone are not sufficient to generalize our findings to all leukocytes. Studies in more types of leukocytes, including normal neutrophils and lymphocytes, are needed. Furthermore, the mechanisms involved in the regulation of apical surface microvillus structure, functions, and mechanics of epithelial cells also need to be investigated. Epithelial cell microvillus formation is regulated by actin filaments and phosphorylated ezrin [34, 35]. In MDCK II kidney epithelial cells, the membrane tension and compressibility modulus of the apical surface are augmented by phosphatidylinositol 4,5-bisphosphate (PIP2)-activated ezrin [24]. On the other hand,

ezrin depletion flattens the MDCK II cells and increases cortical tension and the surface compressibility modulus to some extent [24]. Thus, the relationship between cell surface microvilli, functions, and mechanics in epithelial cells appears to be more complex than that in KG-1 cells. In the future, we would like to further study the relationship between mechanical regulation, functions, and ERM on the surface of various cells, including epithelial cells and normal leukocytes.

In summary, ERMs are necessary to regulate cell adhesion in response to external stimuli in KG-1 cells; ERM dephosphorylation decreases surface stiffness and excludes sialomucins from the attachment site through the loss of the link between cortical actin and transmembrane proteins, resulting in the cell adherence to the substrate. In KG-1 cells, and probably in leukocytes as well, cell adhesion and surface stiffness are closely related, and ERM is a key factor that can control both.

Declarations

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Conflicts of interest

The authors declare that there is no conflict of interest.

Ethics approval

This work does not involve human participants or their data. In this study, we used KG-1 cell line RRID:CVCL_0374, which obtained from RIKEN Bio Resource Center (Ibaraki, Japan).

Informed consent

Not applicable.

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Figure legends

Fig. 1. Stimulated KG-1 cell adhesion to a fibronectin surface in a micro-flow channel. (A) Phase contrast images of KG-1 cells in the fibronectin coated micro-flow channel with or without stimuli. The cells were treated with 100 nM PMA, 10 nM staurosporine (Stauro), or 1.0 nM calyculin A (Cal-A) for 3 h. (B) The adhered cell number of each condition. The adhered cell number was counted from 8 different field views in each condition. ### $p < 0.001$ vs. adhered cell number of control conditions (Dunnett pairwise comparison test).

Fig. 2. Young's modulus of KG-1 cells treated with stimuli. (A) Confocal laser scanning microscopy (CLSM) images of fluorescence labeled F-actin in KG-1 cells. KG-1 cells were treated with or without 2.0 μg/mL of cytochalasin D (CD) for 1.5 h. Bar: 50 μm. (B) Young's modulus of KG-1 cells treated with or without stimuli. The cells were treated with 2.0 μg/mL cytochalasin D (CD), 10 μM Y27632, 100 nM PMA, 10 nM staurosporine (Stauro), or 1.0 nM calyculin A (Cal-A) for 1.5 h. The Young's modulus of cells was measured using the cone probe. The distribution of the Young's modulus is represented by scatterplots. Each point represents the median value of 25 measuring points in each cell, and the Young's modulus in each condition is represented in more than 20 independent cells. The median value of the Young's modulus (kPa) is shown at the top of each plot. ### $p < 0.001$ vs. Young's modulus of the control.

Fig. 3. Actin attenuated KG-1 cell adhesion to fibronectin surface in a micro-flow channel. (A) Phase contrast images of KG-1 cells in the fibronectin coated micro-flow channel with or without actin attenuation reagents. The cells were treated with 2.0 μg/mL cytochalasin D (CD), 10 μM Y27632, or 30 μM Y27632 for 3 h. (B) The adhered cell number of each condition. The adhered cell number was counted from 8 different field views in each condition. # $p < 0.05$ vs. adhered cell number of control conditions. ## $p < 0.01$ vs. adhered cell number of control conditions.

Fig. 4. Immunoblot analysis of phosphorylated ERM in KG-1 cells. KG-1 cell lysates were subjected to SDS-PAGE and immunoblotting with anti-phospho-ERM or anti-β-

actin antibody. The cells were treated with 10 nM PMA, 5.0 μg/mL cytochalasin D (CD), or 30 μM Y27632 for 30 min. In the immunoblotting with anti-phospho-ERM, the lower-molecular-weight band is the phosphorylated moesin and the band above it is phosphorylated ezrin and radixin. The relative amount of phosphorylated ERM in each lane was digitalized as the ratio to the control after normalization with anti-β-actin expression.

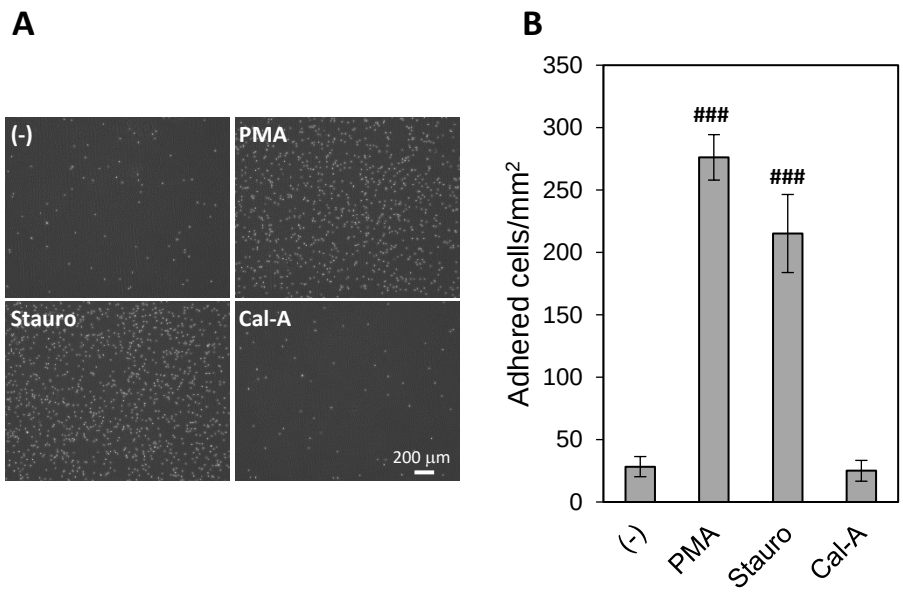


Fig. 1 Kihara et al.

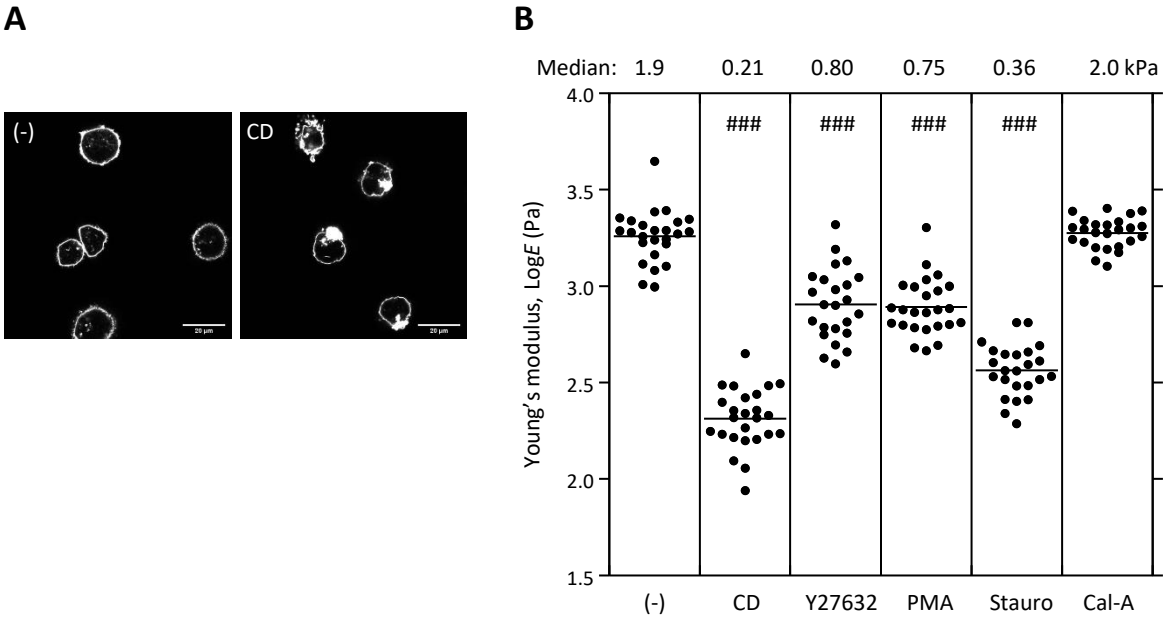


Fig. 2 Kihara et al.

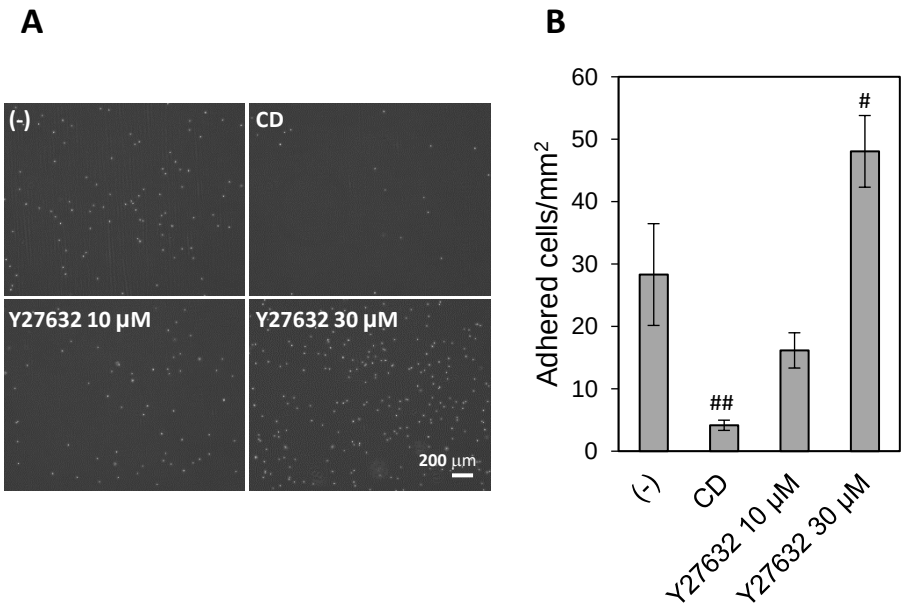


Fig. 3 Kihara et al.

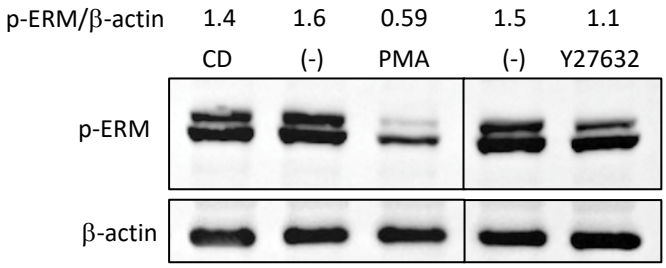
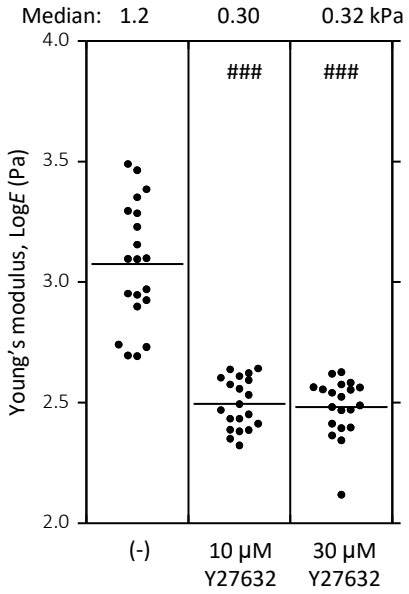


Fig. 4 Kihara et al.



Supplementary Figure S1. Young's modulus of KG-1 cells treated with Y27632. The cells were treated with 10 μM or 30 μM Y27632 for 1.5 h. The Young's modulus of cells was measured using the pyramidal probe. The distribution of the Young's modulus is represented by scatterplots. Each point represents the median value of 25 measuring points in each cell, and the Young's modulus in each condition is represented in 20 independent cells. The median value of the Young's modulus (kPa) is shown at the top of each plot. ### $p < 0.001$ vs. Young's modulus of the control.