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Simultaneous Observation of Calcium Signaling Response and Membrane Deformation due to Localized Mechanical Stimulus in Single Osteoblast-like Cells

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Abstract

Biochemical signals related to a mechanosensory mechanism by which cells sense mechanical stimuli have been gradually clarified by biological approaches such as blocking specific signaling pathways; however, mechanical signals such as deformation/strain, which is transduced into biochemical signals through this mechanism, particularly at the cellular structural component level in a single cell, have not yet been clearly understood. This in vitro study focuses on an intracellular calcium signaling response to an applied localized deformation in a single osteoblast-like MC3T3-E1 cell, and observed localized deformation of a cell membrane and the calcium ion flux from an extracellular medium. The localized deformation was applied to a cell by indenting a microsphere onto the cell membrane using a glass microneedle. The cellular calcium signaling response and cell membrane deformation were simultaneously observed using fluorescent dyes in a vertical section under a confocal laser-scanning microscope with high spatial and temporal resolutions. Our results observed in the vertical section showed that the initiation point of the calcium ion flux is collocated at the displaced microsphere around which stretch membrane deformation was observed.
**Introduction**

It has been proposed that the activities of cells, which are elemental components of living organs and tissues, are modulated by not only biochemical factors but also mechanical factors such as stress and/or strain (van der Meulen and Huiskes, 2002; Sato et al., 2006). It was reported that mechanical stimuli affect the activities of osteoblasts, which are known as bone-forming cells, and regulate various processes such as cell proliferation (Buckley et al., 1988), PGE2 production (Smalt et al., 1997; Fermor et al., 1998), and bone-specific gene expression (Roelofsen et al., 1995). These cellular responses to mechanical stimuli are believed to arise from the transduction mechanism from mechanical signals to biochemical signals, which is called mechanotransduction (Duncan and Turner, 1995). In addition, a mechanosensory mechanism by which cells sense a mechanical stimulus and transduce it to biochemical signals is believed to exist; however, this mechanism is still not clearly understood. Some candidate elemental components playing roles in this mechanism have been proposed, for example, stretch-activated (SA) channels (Naruse et al., 1998) and the cytoskeletal structural system including actin fibers and integrins (Duncan and Turner, 1995; Ingber, 1998; Adachi et al., 2003; Sato et al., 2004, 2007). Moreover, approaches using advanced techniques in molecular biology and biochemistry gradually clarify biochemical signaling pathways downstream of mechanotransduction (Labrador et al., 2003; Mullender et al., 2004).

To clarify this mechanosensory mechanism from the viewpoint of cell biomechanics, it is important to understand mechanical conditions related to this mechanism. Previous *in vitro* experimental studies revealed the characteristics of a cellular response in a controlled mechanical environment. For example, controlled stretching due to a deformation of an elastic substrate (Binderman et al., 1988; Jones et al., 1991) or shear stress induced by extracellular fluid flow (Reich and Frangos, 1993; Klein-Nulend et al., 1997; Jacobs et al., 1998) was applied to cells, and cellular responses to applied stimuli were quantitatively evaluated. In most of these studies, homogeneous deformation or force was applied to a population of cells, so that only the average cellular response
was evaluated. Therefore, to understand mechanical conditions sensed by a cell through the
mechanosensory mechanism, it is indispensable to develop techniques to evaluate these local
mechanical conditions particularly at the cellular structural component level, such as the local
deformation/strain of a cell membrane with the substructures of actin filaments and its cross-linking
proteins just beneath the membrane.

To investigate the relationship between the local deformation of structural components and
cellular responses, a mechanical stimulus should be locally applied to a single cell. A conventional
method of applying local deformation to a single cell is the microperturbation method, which involves
a direct indentation onto the cell membrane using a glass microneedle with a tip diameter of about 1 -
10 μm. This method enables the application of a mechanical stimulus to the targeted single cell.
Therefore, researchers use this method, for example, to evaluate the characteristics of intercellular
signaling communication (Xia and Ferrier, 1992; Guilak et al., 1999). However, these experimental
studies only evaluated the overall characteristics of cellular responses, and local signaling responses
such as calcium ion flux from an extracellular medium due to local membrane deformation have not
been considered.

The objective of this study was to develop an in vitro technique of applying local
deformation to the membrane of a single cell under experimental conditions that enable us to conduct
the simultaneous observation of cell membrane deformation and calcium signaling response in a
vertical section in addition to a conventional horizontal section. Localized deformation was applied to
an osteoblast-like MC3T3-E1 cell by indenting a microsphere onto the cell membrane using a glass
microneedle. The applied localized deformation induced a highly localized response, calcium ion flux
from an extracellular medium, which enabled us to determine its signaling initiation point. A change in
intracellular calcium ion concentration ([Ca^{2+}]), which exists upstream of biochemical signaling
cascades and plays a triggering role in succeeding downstream signals, was observed. The cell
membrane deformation and the change in [Ca^{2+}] were simultaneously observed using a multiple
fluorescent labeling technique and a confocal laser scanning microscope with high spatial and temporal resolutions. From the images observed in vertical sections, the initiation point of the cellular calcium signaling response was determined, and the deformation behavior of the cell membrane around the initiation point was investigated.

Materials and Methods

Cell culture

Osteoblast-like MC3T3-E1 cells (Kodama et al., 1983; Quarles et al., 1992) obtained from the RIKEN BioResource Center (RIKEN BRC) were plated on a glass-bottom culture dish (φ = 35 mm) at a density of 6.0 × 10⁴ cells / dish. The cells were incubated in the α-minimum essential medium (α-MEM, ICN Biomedicals), which contains 10% fetal bovine serum (FBS, ICN Biomedicals), and in 95% air - 5% CO₂ humidified 37°C atmosphere. After 12-hours preincubation after plating, the cells were used in the experiment.

Observation of cell membrane and intracellular calcium ion

After preincubation, the cell membrane and intracellular calcium ions were labeled using fluorescent dyes. The cell membrane was labeled by incubating the cells in Opti-MEM (Invitrogen) containing 20 μM Vybrant DiI (Molecular Probes) for 4 min at 37°C. After rinsing with PBS, the intracellular calcium ions were labeled by incubating the cells in Opti-MEM containing 5 μM Fluo-4-AM (Molecular Probes) for 40 min at 25°C. Finally, after rinsing with PBS, the medium was replaced with α-MEM for observation. Fluorescence images were obtained using a confocal laser scanning microscope (LSM 510, Carl Zeiss) with a 100× oil immersion objective lens. All the obtained images were recorded in PC as intensity data at an eight-bit resolution.

To obtain vertical section images, a galvano-stage unit (Carl Zeiss) was mounted on the microscope stage. Synchronized with laser scanning, the galvano-stage unit moves the glass-bottom
culture dish vertically up and down. This synchronized movement enables us to obtain vertical section images with a high temporal resolution. In this study, we obtained vertical section images with 30 scan lines of 10 μm height in a vertical plane at a rate of 0.25 sec per image.

**Application of localized mechanical stimulus**

To apply localized deformation as a mechanical stimulus to a single cell, the cell membrane was indented with a microsphere adhering to the cell membrane using a glass microneedle. The polybead carboxylate microsphere (Polyscience) with a diameter of 1.0 μm was coated with fibronectin (Morinaga Bioscience Research Center) to enhance its adherence to the cell membrane and placed in the medium during calcium fluorescent indicator loading for 40 min for it to adhere to the cell membrane. Figure 1 shows a schematic of the mechanical stimulus application. The glass microneedle with a 1.0-μm-diameter tip was placed above the microsphere adhering to the cell membrane using a three-dimensional micromanipulator (ONW-135, Narishige), as shown in Fig.1(a). As shown in Fig.1(b), the microneedle was then moved downward to push the microsphere onto the cell membrane and form an indentation. In this tapping process, it was not possible to perfectly control the indentation direction vertically because the roundly polished needle tip was used to tap the round microsphere. Cells with damage in the membrane due to the indentation were discarded by monitoring a change in fluorescence intensity with time; when the membrane was damaged, the intensity rapidly decreased below the basal level, while the intensity in undamaged cells decreased back to the basal level in a few minutes.

**Measurement of strain distribution on cell membrane**

The distribution of strain on the cell membrane due to the application of local deformation was measured by the following method of image analysis (Sato et al., 2007). The displacement field of the fluorescently labeled cell membrane with DiI, a lipophilic membrane stain, was measured by the
image correlation method between the obtained time sequential images using image processing software (Flow-vec 32, Library). Grid points at 10 pixel intervals were then set on the observed region, and triangle finite elements were formed by closing the grid points by considering them as nodal points. To obtain the relatively large displacement vector from the initial state to the state at which the cellular calcium signaling response was observed, all the stepwise displacements measured from each sequential image at 0.25 sec intervals were summed up. That is, the total strains of the triangle elements were obtained by integrating each incremental displacement vector at each node. To evaluate the deformation behavior of the cell membrane, Green’s strain was calculated from the obtained displacement field, and the magnitude and direction of the maximum principal strain were calculated.

Results

Calcium signaling response to applied local deformation observed in horizontal section

Calcium signaling response to the applied local deformation, known as an influx of calcium ions from the extracellular medium through ion channels on the membrane (Xia and Ferrier, 1992; Guilak et al., 1994), was observed in a conventionally observed horizontal plane, as shown in Fig.2. This figure shows the magnified images of the cell around its center within a rectangular region of 28.0×13.5 μm² (512×246 pixels), that is, the entire cell body is not shown. Figure 2(a) shows the initial state of the cell before the mechanical stimulus application, Fig.2(b) shows the state at the time point \( t = 0 \) sec defined immediately before the initiation of the cellular calcium response, and Figs.2(c) and (d) show the states at \( t = 0.23 \) and 0.46 sec, respectively, after the initiation of the calcium signaling response. The upper row shows the cell membrane labeled with Vybrant DiI, the middle row shows [Ca\(^{2+}\)]\text{\textregistered}\text{I} labeled with Fluo 4, and the lower row shows a line profile of Fluo 4 fluorescence intensity. The line profile indicates the distribution of Fluo 4 fluorescence intensity on the line (A - A’) indicated in Fig.2(a).

At the initial state, the fluorescence intensity of Fluo 4 distributed homogeneously in the
entire cell body, as shown in the middle row of Fig. 2(a). At \( t = 0.0 \) sec, as shown in Fig. 2(b), the adhered microsphere was indented onto the cell membrane using the glass microneedle at the point indicated by an arrowhead (m) in the upper row of Fig. 2(b). A white spot due to the microsphere indentation was observed in the fluorescence image of Fluo 4, as shown in the middle row of Fig. 2(b). This spot was also observed in the line profile indicated by an arrowhead (p) in the lower row of Fig. 2(b). Figure 2(c) shows the state at \( t = 0.23 \) sec, at which the microsphere slightly moved in the direction of an arrowhead (n) in the upper row owing to the indentation. At this time point, a local increase in the fluorescence intensity of Fluo 4 was observed in the front region of the translated microsphere, which could be confirmed in the line profile of fluorescence intensity, as indicated by an arrowhead (q) in the lower row of Fig. 2(c). This localized increase in the fluorescence intensity of Fluo 4 could be considered as the initiation point of the cellular calcium signaling response to the applied local deformation. Subsequently, the increase in the fluorescence intensity of Fluo 4 propagated to the adjacent region of the initiation point at \( t = 0.46 \) sec, as indicated by an arrowhead (r) in Fig. 2(d). This result observed in the horizontal section is consistent with that observed in the cell to which a microneedle tip was directly indented and moved horizontally on the cell membrane (Sato et al., 2007). In addition, the transient increase in the intracellular calcium ion concentration due to the mechanical perturbation gradually decreases to the basal level in a few minutes (Adachi et al., 2003; Sato et al., 2007).

**Calcium signaling response to applied local deformation observed in vertical section**

Calcium signaling response to the applied local deformation was observed in a vertical section, as shown in Fig. 3. The observed cell in Fig. 3 is different from that observed in the horizontal section shown in the last section. In this figure, the left column shows the fluorescence images of the cell membrane labeled with Vybrant DiI, the center column shows the fluorescence images of \([\text{Ca}^{2+}]\) labeled with Fluo 4, and the right column shows the analyzed images of Vybrant DiI and Fluo 4 within
a rectangular region of $23.0 \times 10.0 \mu m^2$ (512×224 pixels). In the analyzed images (right column), the binarized image of Vybrant DiI and the contour image of Fluo 4 were superimposed. In addition, to reduce the noise in the obtained image data, data smoothing was carried out, that is, pixel data were averaged within the surrounding 7×7 pixel square. Figure 3(a) shows the initial state before the indentation of the microsphere, Fig.3(b) shows the state at the time point $t = 0$ sec defined immediately before the initiation of the cellular calcium response, and Figs.3(c) and (d) show the states at $t = 0.25$ and 0.50 sec, respectively.

In the initial state shown in Fig.3(a), the fluorescence intensity of Fluo 4 distributed homogeneously in the cytosol, which is the inner area of the cell membrane. At $t = 0.0$ sec, as shown in Fig.3(b), the microsphere was indented onto the cell membrane, and a dimple due to the indentation was observed in the fluorescence image of Vybrant DiI, as indicated by an arrowhead (p) in the left column of Fig.3(b). At this time point, a change in the fluorescence intensity of Fluo 4 was not observed. Figure 3(c) shows the state at $t = 0.25$ sec, at which a local increase in the fluorescence intensity of Fluo 4 was observed underneath the microsphere indentation, as indicated by an arrowhead (q) in the right column of Fig.3(c). Subsequently, the local increase in the fluorescence intensity of Fluo 4 propagated to the entire cytosol region at $t = 0.50$ sec, as shown in black in Fig.3(d).

Figure 4 shows the distribution of the principal strain $\varepsilon_1$ of the cell membrane in the observed vertical section at the time of the initiation of the calcium signaling response. Figures 4(a) and (b) indicate a fluorescence image of the cell membrane labeled with Vybrant DiI and a superimposed image of the Fluo 4 contour and binarized cell membrane images, respectively. The magnitude and direction of the principal strain $\varepsilon_1$ were analyzed in the area indicated by a rectangle in Fig.4(a), and are shown in Figs.4(c) and (d). The initiation point of the local calcium signaling response was collocated at the displaced microsphere, as indicated by an arrow (p) in Fig.4(b). Around the point of the calcium influx from the extracellular medium, as indicated in Fig.4(e) as a magnified image of the
dotted rectangles in Figs. 4(c) and (d), the stretching strain was observed along the cell membrane as shown in Figs. 4(c) and (d). The average magnitude of the principal strain \( \varepsilon_1 \) at the point was evaluated as \( \varepsilon_1 = 0.55 \pm 0.23 \) (Mean \( \pm \) S.D., number of cells \( n = 6 \)), that is a stretching strain.

Discussion

In this study, we developed a novel in vitro technique of applying local deformation to the membrane of a single cell and to simultaneously observe the deformation and calcium signaling response in vertical sections as well as in conventionally observed horizontal sections. In particular, the vertical section images obtained with a high temporal resolution give us significant information about the location and mechanical conditions at the initiation point of the calcium ion influx from the extracellular medium in the vicinity of the applied local deformation.

In previous studies, various characteristics of cellular responses to applied mechanical stimuli were evaluated. For example, the application of strain at magnitudes of 200 - 1000 \( \mu \) strain to osteoblasts via elastic culture substrate stretching induces increases in cell proliferation rate (Brighton et al., 1991), PGE\(_2\) production level (Harell et al., 1977), and bone-specific gene expression level (Roelofsen et al., 1995). The application of a large magnitude of stretching (from 10 to 30 \%) affects the release of reactive oxygen species (Yamamoto et al., 2005), and the application of cyclic stretching induces the downregulation of the expression of HB-GAM, a heparin-binding growth-associated molecule (Liedert et al., 2004). In these studies, changes in surrounding mechanical environmental conditions, such as substrate deformation, were considered as the controlled mechanical stimuli to the cells. However, to clarify the mechanosensory mechanism from the viewpoint of cell biomechanics, the evaluation of the mechanical conditions, such as local cell membrane deformation, which activates the mechanosensory mechanism at the cell structural component level, is indispensable. Therefore, our attempt to directly apply local deformation to the cell membrane and to simultaneously observe the deformation and calcium signaling response are important fundamental steps to gaining insights into
the relationship between the local mechanical conditions and the initiation of cellular responses.

From the observations of vertical sections, the calcium signaling response was locally initiated underneath the cell membrane owing to the indentation of the adhered microsphere. This result may support the hypothesis that the local deformation due to the microsphere indentation is sensed as a mechanical signal and transduced into intracellular biochemical signals through mechanisms such as that involving stretch-activated (SA) channels (Duncan and Misler, 1989; Guharay and Sachs, 1984; Kanzaki et al., 1999) existing in the cell membrane. On the other hand, at the whole-cell level, the cytoskeletal system including actin stress fibers and adhesive protein integrins is believed to play very important roles in the mechanosensory mechanism. Previous reports suggested the importance of the cytoskeletal system in the mechanosensory mechanism (Duncan and Turner 1995; Ingber, 1998). It was proposed that the cytoskeletal structure transmits or even amplifies the mechanical stimulus applied from the extracellular environment into the transducing mechanism. We have also reported that the amount of organized actin structures affects the sensitivity of the cellular calcium signaling response to the mechanical stimulus in osteoblast-like cells (Sato et al., 2004) and suggested the possibility that the aligned actin cytoskeletal structure causes the directional dependence of the response (Adachi et al., 2003). Because the highly localized deformation was applied to the cell in this study, the contribution of the cytoskeletal structure spread in the cell in the mechanosensory mechanism was possibly underestimated in this study. Therefore, to clarify the mechanosensory mechanism, studies at the subcellular level such as this study and those at the cellular level have to be complementarily carried out.

Because mechanical forces, such as tension, in the membrane are supported by the cortex with actin filaments and its cross-linking proteins just beneath the cell membrane, the local deformation was applied to the membrane with these substructures in this study. To label and visualize the cell membrane, we used the DiI, which is a lipophilic membrane stain. However, in the displacement/strain analysis using an image correlation method, it was impossible to extract the
membrane itself automatically from the original images observed. Thus, the displacement/strain analysis was conducted in an entire area using the fluorescent images of the DiI, even though the displacement/strain inside a cell has no meaning but that on the membrane has meaning. After the analysis, we then determined the strain values on the membrane around the area where an initial increase in calcium ion concentration was observed.

To obtain vertical section images, we used the galvano-stage unit mounted on the microscope stage. This unit moves the culture dish with cells vertically up and down at a frequency of 4 Hz; 0.25 sec per one vertical image with 30 scan lines in the range of 10 μm in the vertical direction. This technique enables us to obtain vertical section images with a high temporal resolution. However, because the microneedle is also mounted on the same stage with the dish, a relative motion between the cells and the needle tip is unavoidable. This is one reason why we used a microsphere to locally deform the cell membrane by tapping it using a microneedle. The other reason is that we expected the direct application of the deformation to the membrane using a microsphere; the microsphere was coated with fibronectin that binds to the integrin receptors on the membrane.

Strain on the cell membrane was calculated from the displacement field that was measured using the image correlation method. Image resolutions obtained in the experiment were 0.045 μm/pixel in the horizontal section, and 0.33 μm/slice, each slice was interpolated with 7 pixels, in the vertical direction. Considering that the image correlation method possesses sub-pixel information, the accuracy of the displacement measured in this study could be estimated about 0.1 – 0.2 μm, that is less than about 7 % of the actual microsphere displacement. In the strain analysis, the reference length-measures were set 0.45 μm (10 pixels) in the horizontal section and 0.99 μm (3 slices) in the vertical direction. Thus, the accuracy of the strain measurement in vertical direction was less than that in the horizontal section. To match the accuracy in all directions which is desirable to conduct three-dimensional strain analysis, the limitation of the measuring rate in the vertical direction needs to be improved.
Despite of the several limitations, we demonstrated the quantitative evaluation of strain magnitude at the initiation point of the cellular calcium signaling response to the local membrane deformation in adhered osteoblast-like cells. The quantitative evaluation of mechanical conditions that activate the mechanosensory mechanism has already been conducted; for example, Sokabe et al. (1991) reported that the activation of SA channels depends on the magnitude of tension in the cell membrane, as determined by the micropipette aspiration and patch clamp measurement techniques. However, in their experimental system, suspended cells were used. That is, mechanical conditions such as tension in the cell membrane and the cytoskeletal structure in suspended cells might be different from those in the adhered cells such as osteoblasts. Therefore, the quantitative evaluation of mechanical conditions at the cellular structural component level using cells in the adherent state would be more appropriate for gaining insight the mechanosensory mechanism. Even though our observation was conducted under very simple and limited conditions, it could be considered as the fundamental first step in evaluating mechanical conditions that activate the mechanosensory mechanism. Higher temporal and spatial resolutions than those used in this study are desired to discuss the characteristics of the mechanosensory mechanism in detail.

One of the important future directions of this study is the observation of other major cellular structural components such as cytoskeletal actin fibers and focal adhesion complexes including integrins. In this present study, even though we only focused on the deformation behavior of the cell membrane, the evaluation of the strain magnitude of the membrane at the initiation point of the calcium signaling response possibly provides us valuable information about mechanical conditions that activate SA channels existing in the cell membrane. As discussed previously, the contribution of the cytoskeletal structure to the mechanosensory mechanism is significant. In addition, the microsphere was adhered to the cell membrane through integrin receptors that are believed to connect to the actin cytoskeletal structural system. Thus, the local deformation due to the indentation of the microsphere was transmitted to the cytoskeletal structure and cell membrane. Therefore, the
observation of other major structural components is indispensable in discussing the relationship between the complex mechanical conditions of the cellular structure and the activation of the mechanosensory mechanism.

Conclusions

In this study, a novel technique was proposed for the simultaneous observation of the calcium signaling response and membrane deformation in a single cell due to a localized mechanical stimulus. In particular, vertical section images were obtained with a high temporal resolution, enabling us to observe the initiation point of the calcium ion influx from the extracellular medium. Combined with an image correlation method with a high precision for displacement/strain analysis, the future applications of the novel technique to the research in the field of mechanobiology are greatly expected.

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Figures

**Fig.1:** Schematic of local deformation application to single osteoblast-like cell. The fibronectin-coated microsphere ($\phi = 1.0 \, \mu m$) adhering to the cell membrane was indented downward onto the cell using a glass microneedle with a 1.0-\(\mu\)m-diameter tip attached to a three-dimensional micromanipulator.
Fig. 2: Observed images of cell membrane and intracellular calcium ion in two-dimensional horizontal section using confocal laser scanning microscope. The upper row shows the cell membrane labeled with Vybrant DiI, the middle row shows the intracellular calcium ion concentration labeled with Fluo 4, and the lower row shows the line profile of the fluorescence intensity of Fluo 4. The microsphere was indented at the point indicated by arrowheads (m) and (p) at $t = 0$ sec. Owing to the indentation, the microsphere moved in the direction of an arrowhead (n) at $t = 0.23$ sec. At this time point, a local increase in the fluorescence intensity of Fluo 4 was observed in the region adjacent to the microsphere, as indicated by an arrowhead (q). At $t = 0.46$ sec, the increase in fluorescence intensity propagated to the adjacent region, as indicated by an arrowhead (r).
(a) Initial

(b) $t = 0.0$

(c) $t = 0.25$

(d) $t = 0.5$ (sec)

Fluorescence image of Vybrant DiI (cell membrane)
Fluorescence image of Fluo 4 (intracellular calcium ion)
Superimposed images of contour image (Fluo 4) and binarized image (Vybrant DiI)

**Fig.3**: Observed images of cell membrane and intracellular calcium ion in vertical section. The left column shows the cell membrane labeled with Vybrant DiI, the middle column shows the intracellular calcium ion concentration labeled with Fluo 4, and the right column shows the superimposed images of the contour image of Fluo 4 and the binarized image of Vybrant DiI. The microsphere was indented at the point indicated by an arrowhead (p) at $t = 0$ sec. At $t = 0.25$ sec, a local increase in the fluorescence intensity of Fluo 4 was observed underneath the microsphere, as indicated by an arrowhead (q) in the superimposed image (c).
Fig. 4: Distribution of principal strain $\varepsilon_1$ around initiation point of calcium signaling response observed in vertical section. (a) and (b) indicate the fluorescence image of the cell membrane (Vybrant DiI) and the superimposed image of the Fluo 4 contour image and the binarized image of the cell membrane (Vybrant DiI), respectively. The initiation point of the calcium signaling response was detected in the region adjacent to the indented microsphere, as indicated by an arrowhead (p) in Fig. 4(b). Figures 4(c) and (d) indicate the magnitude and directions of the principal strain around the initiation point of the calcium signaling response, respectively. The analyzed area is indicated by a rectangle in (a). Stretching strain was observed along the cell membrane, as shown in (d). The average magnitude of the strain was evaluated as $\varepsilon_1 = 0.55 \pm 0.23$ (mean $\pm$ S.D., number of cells $n = 6$).