Visualization of Ins(1,4,5)P_3 dynamics in living cells: two distinct pathways for Ins(1,4,5)P_3 generation following mechanical stimulation of HSY-EA1 cells

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Summary

In the present study, the contribution of inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3] generation on the mechanical-stimulation-induced Ca^{2+} response was investigated in HSY-EA1 cells. Mechanical stimulation induced a local increase in the cytosolic concentration of Ins(1,4,5)P_3 ($[IP_3]_i$), as indicated by the Ins(1,4,5)P_3 biosensor LIBRAvIII. The area of this increase expanded like an intracellular Ins(1,4,5)P_3 wave as $[IP_3]_i$ increased in the stimulated region. A small transient $[IP_3]_i$ increase was subsequently seen in neighboring cells. The phospholipase C inhibitor U-73122 abolished these Ins(1,4,5)P_3 responses and resultant Ca^{2+} releases. The purinergic receptor blocker suramin completely blocked increases in $[IP_3]_i$ and the Ca^{2+} release in neighboring cells, but failed to attenuate the responses in mechanically stimulated cells. These results indicate that generation of Ins(1,4,5)P_3 in response to mechanical stimulation is primarily independent of extracellular ATP. The speed of the mechanical-stimulation-induced $[IP_3]_i$ increase was much more rapid than that induced by a supramaximal concentration of ATP (1 mM). The contribution of the Ins(1,4,5)P_3-induced Ca^{2+} release was larger than that of Ca^{2+} entry in the Ca^{2+} response to mechanical stimulation in HSY-EA1 cells.

Key words: Mechanical stimulation, Inositol (1,4,5)-trisphosphate, Ca^{2+} response, FRET, Fluorescent biosensor, Spatial dynamics

Introduction

Mechanical stimuli, such as shear stress, stretch and touch, elicit increases in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in living cells. A touch stimulation applied to a single cell induces a transient $[Ca^{2+}]_i$ increase within that cell. This Ca^{2+} response then propagates to neighboring cells as an intercellular Ca^{2+} wave. These sequential responses have been observed in various cell types, including epithelial cells (Churchill et al., 1996; Enomoto et al., 1994; Sanderson et al., 1990), endothelial cells (Demer et al., 1993; Moerenhout et al., 2001b) and osteoblastic cells (Xia and Ferrier, 1992).

Mechanical stimulation induces Ca^{2+} entry through mechanosensitive channels on the membrane surface, and this entry is thought to be an important pathway for mechanical-stimulation-induced Ca^{2+} responses (Boitano et al., 1994; Guharay and Sachs, 1984; Hamill and McBride, 1996; Stalmans and Himpens, 1997). In addition, mechanical stimulation induces ATP release, which has been implicated in the propagation of intercellular Ca^{2+} waves (Enomoto et al., 1992; Enomoto et al., 1994; Osipchuk and Cahalan, 1992). ATP induces inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3 or IP_3]-induced Ca^{2+} release from intracellular Ca^{2+} stores through the activation of P2Y purinergic receptors. This, in turn, suggests the involvement of Ins(1,4,5)P_3-induced Ca^{2+} release in Ca^{2+} responses in mechanically stimulated cells, owing to the autocrine actions of ATP. In fact, Ins(1,4,5)P_3-induced Ca^{2+} release reportedly contributes to mechanically stimulated cell responses in human submandibular gland (HSG) cells (Ryu et al., 2010) and other cell types (Boitano et al., 1992; Hansen et al., 1993; Moerenhout et al., 2001b; Sanderson et al., 1990). However, neither the actual changes in the cytosolic Ins(1,4,5)P_3 concentration ($[IP_3]_i$), nor the relative contribution of Ins(1,4,5)P_3-induced Ca^{2+} release in mechanical-stimulation-induced Ca^{2+} responses have been clarified.

Recently, we developed a FRET-based Ins(1,4,5)P_3 biosensor, LIBRA, that consists of the ligand-binding domain of the Ins(1,4,5)P_3 receptor and a pair of fluorescent proteins, cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) (Tanimura et al., 2004). LIBRA variants such as pH-resistant LIBRA (LIBRAvIII) and Ins(1,4,5)P_3-insensitive LIBRA (LIBRAvN) were also developed, as was a method for quantifying $[IP_3]_i$ in single living cells (Tanimura et al., 2009). These new methods have enabled the investigation of mechanical-stimulation-induced Ins(1,4,5)P_3 dynamics and their contribution to Ca^{2+} responses.

Agonist-induced Ca^{2+} and Ins(1,4,5)P_3 responses are well characterized in the HSY human parotid cell line (HSY-EA1 cells) (Tanimura et al., 2009). In this study, the mechanical-stimulation-induced spatiotemporal dynamics of Ins(1,4,5)P_3 were examined in HSY-EA1 cells. Mechanical stimulation generated an intracellular gradient of Ins(1,4,5)P_3 in stimulated cells and intercellular waves of Ins(1,4,5)P_3 in neighboring cells. Unlike the ATP-mediated Ins(1,4,5)P_3 response in neighboring cells, mechanically stimulated cells showed a strong, rapid increase in $[IP_3]_i$ in an ATP-independent manner. These data clarify that this new ATP-independent pathway of Ins(1,4,5)P_3 generation has an important role in Ca^{2+} responses in mechanically stimulated cells.

Results

Mechanical-stimulation-induced spatiotemporal dynamics of Ca^{2+} and Ins(1,4,5)P_3 in HSY-EA1 cells

When an HSY-EA1 cell was poked with a glass micropipette, the Ca^{2+} response began at the stimulated region (Fig. 1A,B,

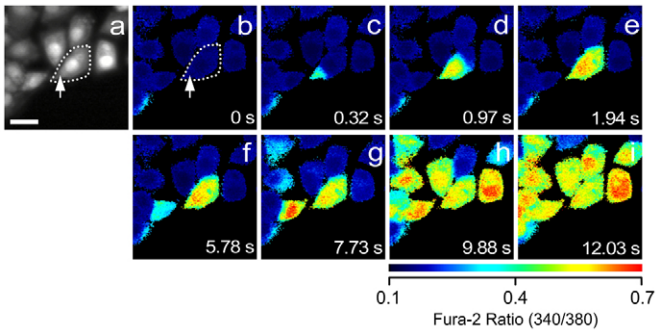

Fig. 1. Mechanical-stimulation-induced Ca^{2+} responses in HSY-EA1 cells. Fura-2-loaded cells were mechanically stimulated with a glass micropipette. (**A**) Fura-2 fluorescence image at 380 nm excitation. Outline indicates the mechanically stimulated cell. (**B-I**) Excitation ratio (340/380 nm) images. Arrow, position of mechanical stimulation. Data shown are typical of six experiments. Scale bar: 10 µm.

arrowhead) and spread throughout the cell within 2 seconds (Fig. 1B-E). Subsequent Ca^{2+} responses propagated from the mechanically stimulated cell to neighboring cells as an intercellular Ca^{2+} wave (Fig. 1F–I). To examine the involvement of Ins(1,4,5)P_3 in these Ca^{2+} responses, LIBRAvIII and Fura-2 were employed to simultaneously monitor changes in $[IP_3]_i$ and $[Ca^{2+}]_i$. An increase in the LIBRAvIII emission ratio (R/R_0) was detected initially around the stimulated region (Fig. 2A). This increase was always higher than that at distant regions, such that an intracellular gradient of $[IP_3]_i$ emerged and expanded like an Ins(1,4,5)P_3 wave (Fig. 2; supplementary material Movie 1). Fluorescence recovery after photobleaching (FRAP) revealed that the LIBRAvIII fluorescence in the photobleached area did not recover for at least 60 seconds (supplementary material Fig. S1), excluding the effect of LIBRAvIII diffusion in this wave-like response. A similar increase in the LIBRAvIII emission ratio after mechanical stimulation was observed concomitantly with the Ca^{2+} response (21 of 21 cells responded). In this time resolution, the onset of the increase in the LIBRAvIII ratio and that of the Ca^{2+} response were detected simultaneously.

We next examined the propagation of mechanical-stimulation-induced Ins(1,4,5)P_3 responses from the stimulated cell to the neighboring cells. Mechanical stimulation initially induced a large transient increase in the LIBRAvIII emission ratio in the stimulated cell. A small transient increase in the LIBRAvIII emission ratio was subsequently seen in the neighboring cells where the propagation of Ca^{2+} responses was observed (Fig. 3A,B; supplementary material Movie 2). The propagation of Ca^{2+} responses was detected shortly (approximately one frame) after increases in the LIBRAvIII emission ratio. Pretreatment of cells with 10 µM U-73122, a phospholipase C (PLC) inhibitor, abolished increases in the LIBRAvIII emission ratio in both mechanically stimulated and neighboring cells, whereas a small Ca^{2+} response was observed in the stimulated cells (Fig. 3C). An ionomycin-induced $[Ca^{2+}]_i$ increase had no effect on the LIBRAvIII emission ratio (Fig. 3E). The emission ratio of LIBRAvN, an Ins(1,4,5)P_3-insensitive variant of LIBRAvIII (Tanimura et al., 2009), did not increase with the mechanical-stimulation-induced Ca^{2+} response (Fig. 3F). These results indicate that changes in the LIBRAvIII emission ratio actually reflect changes in $[IP_3]_i$ in living HSY-EA1 cells.

Based on the assumption that the resting $[IP_3]_i$ of HSY-EA1 cells is ~18 nM (Tanimura et al., 2009), a quantitative analysis indicated that the maximal estimated increases in $[IP_3]_i$ in mechanically stimulated and neighboring cells were 303±31 and 123±24 nM, respectively (Fig. 3G). The times required to reach 50% of the maximal change in the LIBRAvIII emission ratio ($t_{1/2}$) in the stimulated and neighboring cells were 4.3±0.3 and 15±3.7 seconds, respectively (Fig. 4). Pretreatment of cells with 100 µM suramin, a nonspecific purinergic receptor blocker, abolished the mechanical-stimulation-induced $[IP_3]_i$ increases in neighboring cells (Fig. 3D), clearly indicating that the propagation of Ins(1,4,5)P_3 and Ca^{2+} responses were primarily mediated by purinergic receptors. By contrast, suramin had no effect on the extent of the increase in $[IP_3]_i$ (Fig. 3G) or on the $t_{1/2}$ for the maximal Ins(1,4,5)P_3 response (Fig. 4) in mechanically stimulated cells. These data strongly suggest that mechanical stimulation induces Ins(1,4,5)P_3 generation through an ATP-independent pathway in HSY-EA1 cells.

Mechanism of mechanical-stimulation-induced Ca^{2+} responses in HSY-EA1 cells

We next examined the contribution of ATP-dependent and -independent Ins(1,4,5)P_3 generation pathways to mechanical-stimulation-induced Ca^{2+} responses. Cells stimulated under various experimental conditions (Fig. 5; supplementary material Figs S2-

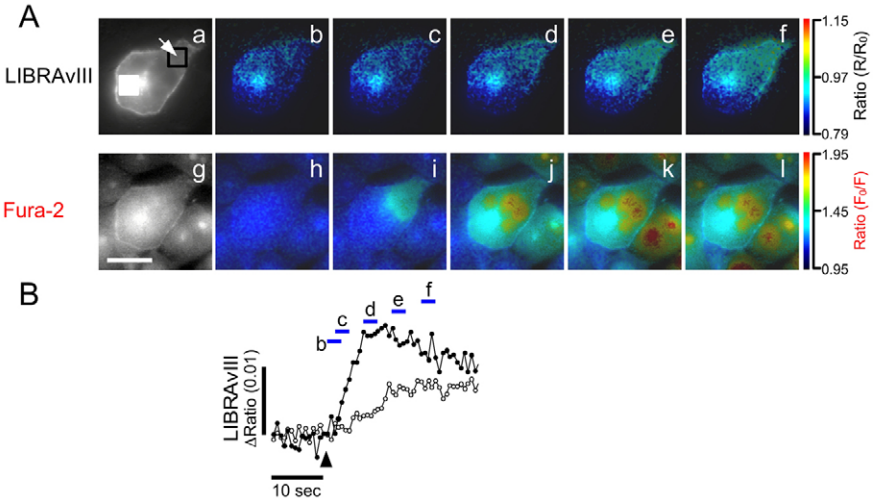

Fig. 2. Mechanical-stimulation-induced Ins(1,4,5)P_3 responses in an HSY-EA1 cell. (**A**, upper panels) Fluorescence image (a) and normalized emission ratio (R/R_0) images (b-f) of LIBRAvIII after mechanical stimulation. (Lower panels) Fura-2 fluorescence image at 380 nm excitation (g) and normalized fluorescence ratio (F_0/F) images (h-l) of Fura-2 after mechanical stimulation. Arrow, position of mechanical stimulation. Panels (b-f) show images at the times indicated by the corresponding letters in panel B. (**B**) Mechanical-stimulation-induced changes in emission ratio (C/Y channels) of LIBRAvIII at the areas indicated by the open square (●) and white square (○) in image a of A. Mechanical stimulation was applied to the point indicated by the arrow in a at the time indicated by the arrowhead in panel B. Data shown are typical of typical of ten experiments. Scale bar: 5 µm.

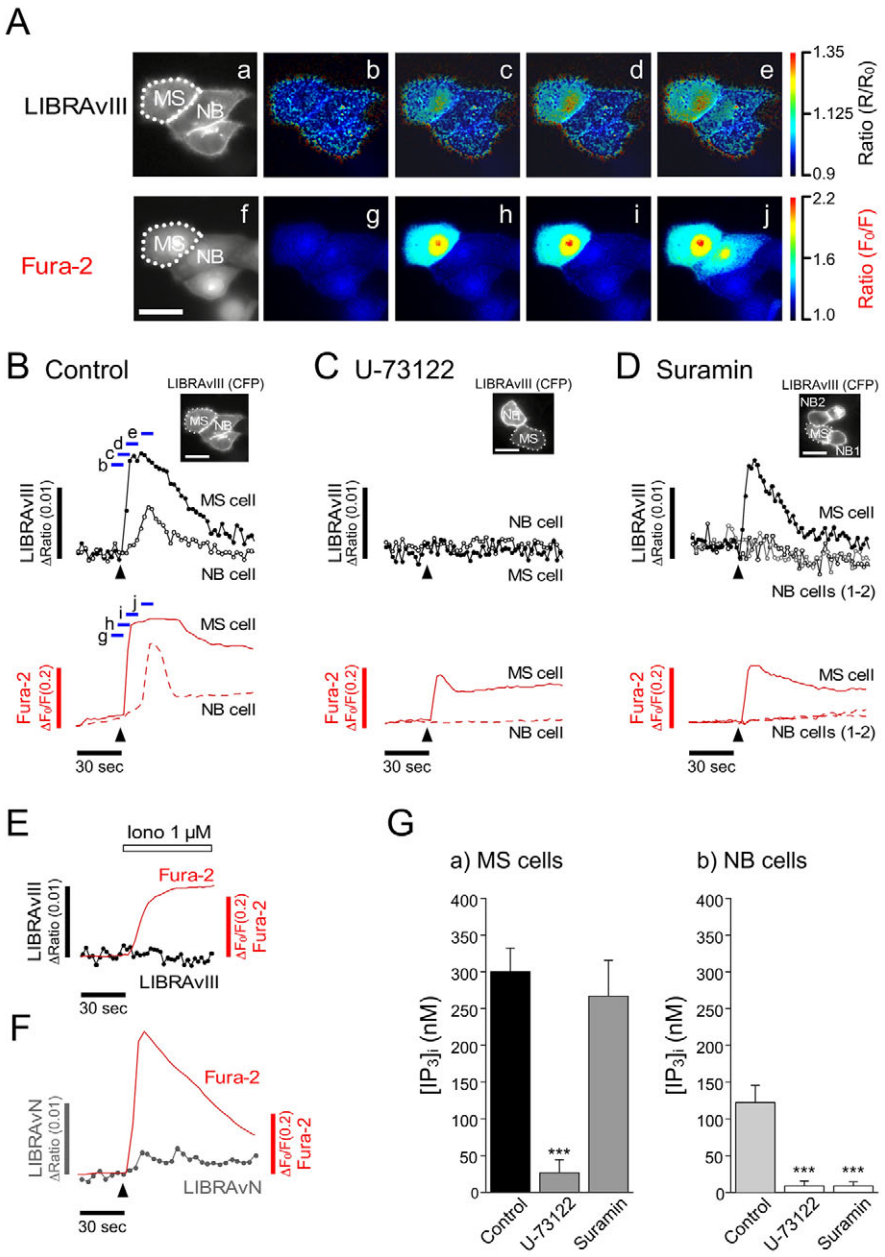

Fig. 3. Effect of U-73122 and suramin on Ins(1,4,5)P_3 responses in mechanically stimulated cells (MS cells) and neighboring cells (NB cells). (**A**, upper panels) Fluorescence image (a) and normalized emission ratio (R/R_0) images (b-e) of LIBRAvIII in an MS or NB cell after mechanical stimulation. (Lower panels) Fura-2 fluorescence image at 380 nm excitation (f) and fluorescence ratio (F_0/F) images (g-j) of Fura-2 after mechanical stimulation. Panels b-e and panels g-j show images at the times indicated by corresponding letters in panel B. Scale bars: 10 μm. Result shown is typical of 21 experiments. (**B-D**) Mechanical-stimulation-induced changes in the ratio of LIBRAvIII (upper panel) and Fura-2 (lower panel) in an MS or NB cell (inset). C and D show the effects of pretreatment with 10 μM U-73122 for 10 minutes (C) or with 100 μM suramin for 20 minutes (D) before stimulation. Arrowhead, time of mechanical stimulation. (**E**) Effect of ionomycin (Iono) on the emission ratio of LIBRAvIII (C/Y channels, closed circles), and the fluorescence ratio of Fura-2 (F_0/F, red line). Horizontal bar at top of the panel, presence of 1 μM Iono. (**F**) Effect of mechanical stimulation on the emission ratio of LIBRAvN (C/Y channels, gray closed circles), and the fluorescence ratio of Fura-2 (F_0/F, red line). Arrowhead, time of mechanical stimulation. (**G**) Maximal $[IP_3]_i$ increases in MS cells (a) and NB cells (b) in the presence or absence of 10 μM U-73122 or 100 μM suramin. Values are means ± s.e.m. of 7-21 cells. ***P<0.001 compared with control.

S5) revealed that an absence of extracellular Ca^{2+} reduced the mechanical-stimulation-induced Ca^{2+} response only slightly in both stimulated and neighboring cells (Fig. 5A,B,I,J). In the mechanically stimulated cells, pretreatment with U-73122 reduced Ca^{2+} responses to ~50% of the control in the presence of extracellular Ca^{2+}, and abolished the response in the absence of extracellular Ca^{2+} (Fig. 5C,D,I). Suramin also reduced Ca^{2+} responses to ~50%. The remaining suramin-resistant Ca^{2+} response was observed even in the absence of extracellular Ca^{2+} (Fig. 5E,F,I), confirming the involvement of ATP-independent Ca^{2+} release. In the neighboring cells, Ca^{2+} responses were completely blocked by U-73122, suramin and an ATP diphosphohydrolase apyrase (Fig. 5C-F,J; supplementary material Fig. S4). The gap junction blockers 1-octanol (0.5 mM) (Toyofuku et al., 1998) and 16-doxyl-stearic acid (16-DSA, 5 μM) (Burt, 1989) failed to block Ca^{2+} responses in mechanically stimulated or neighboring cells (Fig. 5G,J). These results clearly indicate that Ca^{2+} responses in the neighboring cells were primarily mediated by ATP-dependent Ca^{2+} release via purinergic receptors. As expected, a small Ca^{2+} response was observed only in the mechanically stimulated cells under conditions in which the Ca^{2+} store was depleted by thapsigargin (ThG) (Fig. 5H-J; supplementary material Fig. S5).

ATP-induced Ins(1,4,5)P_3 and Ca^{2+} dynamics in HSY-EA1 cells

Next, ATP-induced changes in $[IP_3]_i$ were examined in HSY-EA1 cells. The increase in the LIBRAvIII emission ratio after application of 10 μM ATP was relatively slow, and was observed evenly throughout the cell (Fig. 6A; supplementary material Movie 3). An intracellular $[IP_3]_i$ gradient was not observed in ATP-stimulated cells. Consistent with our previous report, low concentrations of ATP (3-10 μM) induced Ca^{2+} oscillations and concomitant $[IP_3]_i$ oscillatory changes (Fig. 6B); high concentrations of ATP (>30 μM) induced peak-plateau-type responses of Ins(1,4,5)P_3 and Ca^{2+}

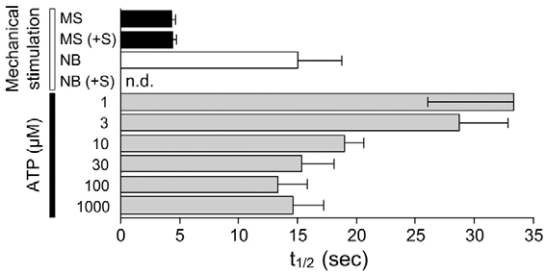

Fig. 4. Speed of Ins(1,4,5)P_3 responses after mechanical stimulation and ATP stimulation. Mechanical stimulation or ATP at various concentrations was applied in the presence (+S) or absence of suramin, and the time required to reach 50% of the maximal increase in ratio (C/Y channels) of LIBRAvIII ($t_{1/2}$) was noted. Values are means ± s.e.m. of 6-21 cells. n.d., not detected; MS, mechanically stimulated cells; NB, neighboring cells.

(Tanimura et al., 2009). There was a large variability in ATP sensitivity among individual HSY-EA1 cells. The initial ATP (>30 μM)-induced $[IP_3]_i$ and $[Ca^{2+}]_i$ increases were detected between 2 and 20 seconds, with average onsets at 7±1 and 8±1 seconds (n=28), respectively. Stimulation with a high concentration of ATP (100 μM) induced no change in the LIBRAvN emission ratio (Fig. 6C).

ATP induced a concentration-dependent increase in $[IP_3]_i$, with a 50% effective concentration (EC_{50}) of ~5.9 μM (Fig. 6D). A similar EC_{50} value of ATP for Ca^{2+} responses was previously reported in rat submandibular gland ductal cells (Amsallem et al., 1996; Park et al., 1997; Turner et al., 1997). Figure 4 summarizes the $t_{1/2}$ values for the maximal $[IP_3]_i$ response to mechanical or ATP stimulation. The ATP-induced $t_{1/2}$ decreased as the ATP concentration increased. The $t_{1/2}$ value at high concentrations of ATP (30-1000 μM) was much larger than that of mechanically stimulated cells and was similar to that in neighboring cells.

Discussion

Mechanical-stimulation-induced ATP-dependent and ATP-independent Ins(1,4,5)P_3 generation in HSY-EA1 cells

In this study, the FRET-based Ins(1,4,5)P_3 biosensor LIBRAvIII was used to monitor changes in $[IP_3]_i$ during mechanical-stimulation-induced Ca^{2+} responses and subsequent propagation of the Ca^{2+} response to neighboring cells. The results of this study provide the first direct demonstration of the dynamics of Ins(1,4,5)P_3 and its contribution to Ca^{2+} responses after mechanical stimulation.

Suramin failed to block the generation of Ins(1,4,5)P_3 and release of Ca^{2+} in mechanically stimulated cells, whereas the generation of suramin-resistant Ins(1,4,5)P_3 and subsequent release of Ca^{2+} were completely blocked by the PLC inhibitor U-73122. These results indicate that Ca^{2+} release in mechanically stimulated cells was induced via an ATP-independent mechanism of Ins(1,4,5)P_3 generation. By contrast, the propagation of Ins(1,4,5)P_3 and Ca^{2+} responses was completely blocked by suramin or apyrase. Mechanical-stimulation-induced propagation of the intercellular Ca^{2+} wave was observed in the absence of extracellular Ca^{2+}, but was completely blocked when intracellular Ca^{2+} stores were depleted by ThG. Thus, the Ca^{2+} responses in neighboring cells were primarily induced by Ca^{2+} release via ATP-dependent generation of Ins(1,4,5)P_3. Although in some cell types gap junctions reportedly contribute to the intercellular Ca^{2+} wave in neighboring cells (Boitano et al., 1992; Charles et al., 1992; Felix et al., 1998; Hansen et al., 1995; Sneyd et al., 1995; Strahonja-Packard and Sanderson, 1999), the gap junction inhibitors 1-octanol and 16-DSA had no effect on Ca^{2+} responses in neighboring cells in the present study.

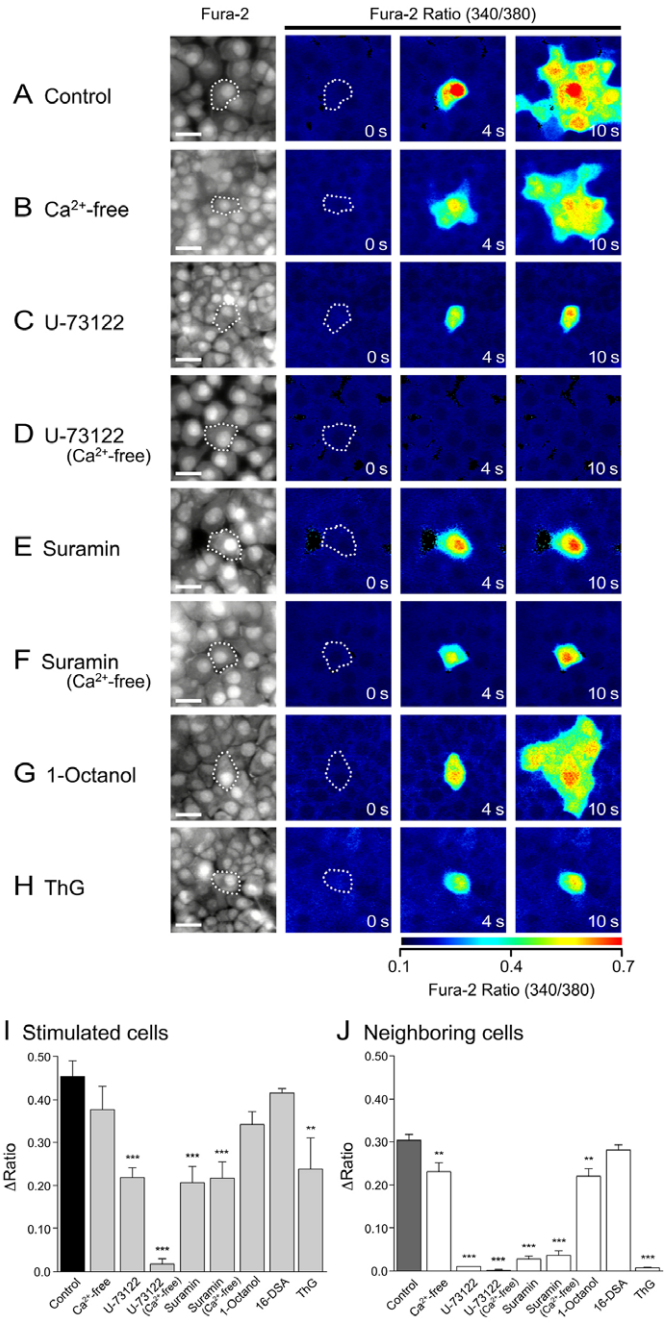

Fig. 5. Ca^{2+} responses of mechanically stimulated cells and neighboring cells in various experimental conditions. Black and white images, fluorescence at 380 nm. Pseudocolor images, excitation ratio (340/380 nm) images of Fura-2. (**A-H**) Control (A), Ca^{2+}-free (B), 10 μM U-73122 (C), 10 μM U-73122 (Ca^{2+}-free; D), 100 μM suramin (E), 100 μM suramin (Ca^{2+}-free; F), 0.5 mM 1-octanol (G), 1 μM thapsigargin (ThG; H). Dotted lines indicate mechanically stimulated cells. Scale bars: 10 μm. (**I**,**J**) Mechanical-stimulation-induced increase in excitation ratio (340/380 nm) of Fura-2 in various experimental conditions in stimulated cells (I) and neighboring cells (J). Values are means ± s.e.m. of 5-18 experiments. **P<0.01 and ***P<0.001 compared with control.

Given that intercellular Ca^{2+} waves are mediated by released ATP, we anticipated that ATP would have autocrine actions on the mechanical-stimulation-induced Ins(1,4,5)P_3 generation and subsequent Ca^{2+} release in stimulated cells. However, the data shown here indicate that contributions of the ATP-mediated pathway to the responses of mechanically stimulated cells were negligible.

Many groups have reported that mechanical stimulation induces Ca^{2+} entry via mechanosensitive channels (Boitano et al., 1994; Guharay and Sachs, 1984; Hamill and McBride, 1996; Stalmans and Himpens, 1997). In the present study, the Ca^{2+} response after pretreatment with ThG indicated that Ca^{2+} entry contributes to the mechanical-stimulation-induced Ca^{2+} response. A Ca^{2+} response was observed in stimulated cells in the absence of extracellular Ca^{2+}, with increases in $[Ca^{2+}]_i$ comparable to those in the presence of extracellular Ca^{2+}. This mechanical-stimulation-induced increase was higher than that observed in ThG-treated cells, indicating that the contribution of the ATP-independent Ca^{2+} release was larger than that of Ca^{2+} entry.

Suramin- or apyrase-resistant Ca^{2+} responses in mechanically stimulated cells have been reported in mammary epithelial cells (Enomoto et al., 1994), airway epithelial cells (Homolya et al., 2000) and pulmonary artery epithelial cells (Moerenhout et al., 2001a). These Ca^{2+} responses have also been shown to be reduced by U-73122 pretreatment (Moerenhout et al., 2001b). Although Ca^{2+} entry is emphasized in these previous reports, ATP-independent PLC-activation might also be involved in these cell types.

Mechanical-stimulation-induced Ins(1,4,5)P_3 changes in HSY-EA1 cells

In the present study, locally generated Ins(1,4,5)P_3 spread only to a limited area, so that an intracellular $[IP_3]_i$ gradient emerged in the relative ratio images. Because Ins(1,4,5)P_3 has been thought to be a rapid, long-distance messenger compared with Ca^{2+} (Allbritton et al., 1992; Kasai, 1995; Kasai and Petersen, 1994), intracellular $[IP_3]_i$ gradients were an unexpected result in this time resolution. Furthermore, the area of increase in $[IP_3]_i$ expanded like an Ins(1,4,5)P_3 wave. This wave-like Ins(1,4,5)P_3 probably reflects the rate of increase in Ins(1,4,5)P_3 at the mechanically stimulated point, in addition to the diffusion component of Ins(1,4,5)P_3. To examine the details of the relationship between the intracellular Ins(1,4,5)P_3 wave and the Ca^{2+} wave, further improvements in Ins(1,4,5)P_3 biosensors and analytic methods with higher time resolutions will be necessary.

Mechanism of mechanical-stimulation-induced Ins(1,4,5)P_3 generation in stimulated cells

The mechanism of Ins(1,4,5)P_3 generation in mechanically stimulated cells remains unknown, although one possibility is that these cells release unknown extracellular messengers. For instance, Enomoto et al. showed that mechanical stimulation causes the release of UDP, and that the UDP-induced response is not blocked by suramin (Enomoto et al., 1994). If this is the case, these extracellular messengers should induce Ca^{2+} responses in neighboring cells. In the present study, however, suramin completely blocked the propagation of intercellular waves of Ins(1,4,5)P_3 and Ca^{2+} from the mechanically stimulated cells. In addition, the speed of the mechanical-stimulation-induced $[IP_3]_i$ increase was far more rapid than that induced by a supramaximal concentration of ATP (1 mM). Thus, unknown extracellular messengers are unlikely to contribute to mechanical-stimulation-induced Ins(1,4,5)P_3 generation in HSY-EA1 cells.

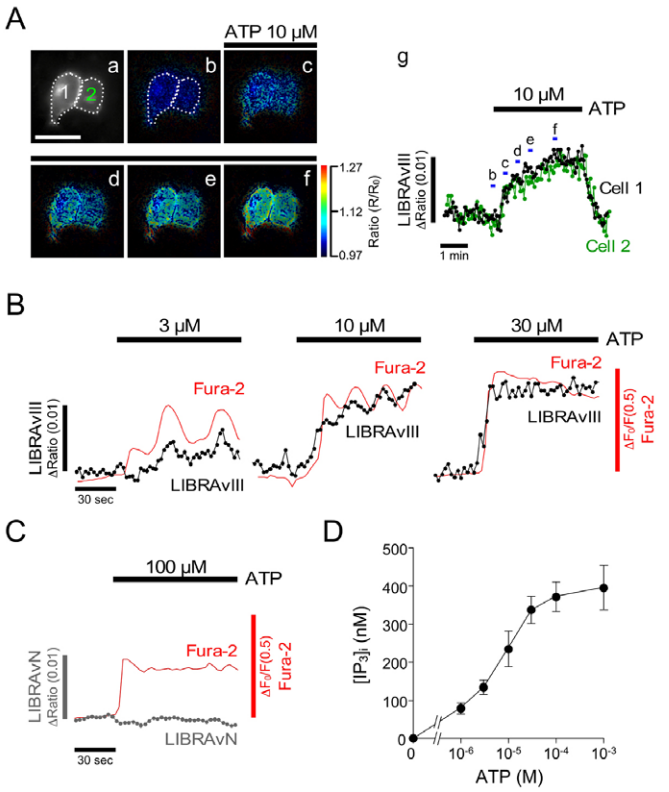

Fig. 6. ATP-induced Ins(1,4,5)P_3 responses in HSY-EA1 cells. (**A**) Fluorescence image (a) and normalized emission ratio (R/R_0) images (b-f) of LIBRAvIII before and after stimulation with 10 μM ATP. (g) ATP-induced changes in the emission ratio (C/Y channels) of LIBRAvIII. Panels (b-f) show images at the times indicated by the corresponding letters in panel (g). The horizontal bar at the top of the panel indicates the presence of 10 μM ATP. Scale bar: 10 μm. Result shown is representative of ten cells. (**B**) Changes in the ratios of LIBRAvIII (C/Y channels, closed circles) and Fura-2 (F_0/F, red lines) after stimulation with various concentrations of ATP. Horizontal bars at the panel top indicate the presence of the indicated concentrations of ATP. (**C**) Effect of ATP on the emission ratio (C/Y channels) of LIBRAvN (C/Y channels, gray closed circles) and the fluorescence ratio of Fura-2 (F_0/F, red line). Horizontal bar at top of the panel indicates the presence of 100 μM ATP. (**D**) Increase in $[IP_3]_i$ with various concentrations of ATP. Values are means ± s.e.m. of six to ten cells.

Ryan et al. reported that mechanical distention, such as stretch stimulation, activates PLC by increasing $[Ca^{2+}]_i$ via Ca^{2+} entry (Ryan et al., 2000). So far, 12 different PLC isozymes have been identified (Berridge, 1993; Hwang et al., 2005; Nakahara et al., 2005; Noh et al., 1995; Rhee and Choi, 1992), one of which, PLC-∂1, is activated by $[Ca^{2+}]_i$ increases through Ca^{2+} entry (Kim et al., 1999). In addition, ionomycin-induced $[Ca^{2+}]_i$ increases cause the generation of Ins(1,4,5)P_3 in PLC-∂1-overexpressing PC12 cells. However, in the present study, ionomycin did not induce Ins(1,4,5)P_3 generation in HSY-EA1 cells. Furthermore, mechanical-stimulation-induced Ca^{2+} release was observed even in the absence of extracellular Ca^{2+}. Therefore, mechanical-stimulation-induced Ins(1,4,5)P_3 generation cannot be attributed to PLC activation through Ca^{2+} entry, at least in the present experiment in HSY-EA1 cells.

Mechanical-stimulation-induced PLC activation has been reported in renal and small cerebral arteries (Narayanan et al., 1994 and Osol et al., 1993), and potentially involves the activity of G-protein-coupled receptors (GPCRs) (Mederos y Schnitzler et al., 2008). Ligand-independent activation of orphan GPCRs and their interaction with non-orphan GPCRs was recently shown (Levoye et al., 2006). These studies suggest that ligand-independent GPCR activation underlies mechanical-stimulation-induced Ins(1,4,5)P_3 generation in HSY-EA1 cells. Despite the attractiveness of this model, further experiments are needed to test its validity.

Mechanism of the mechanical-stimulation-induced intercellular Ins(1,4,5)P_3 wave

Mechanical-stimulation-induced intercellular Ca^{2+} waves due to ATP release have been observed in various cell types. The present study demonstrates for the first time that mechanical stimulation induces intercellular propagation of Ins(1,4,5)P_3 responses by ATP release. These Ins(1,4,5)P_3 responses were quantitatively analyzed using a method established in a previous report (Tanimura et al., 2009). The estimated $[IP_3]_i$ in neighboring cells after mechanical stimulation was comparable to that induced by exposure to 3 μM ATP. Similar levels of ATP release have been observed after mechanical stimulation of Jurkat cells (Corriden et al., 2007), whereas higher levels have been reported in retinal astrocytes (Newman, 2001). However, the $t_{1/2}$ for the maximal increase in $[IP_3]_i$ in neighboring cells was similar to that induced by high concentrations of ATP (>30 μM). We hypothesize that the initial level of released ATP is relatively high (>30 μM), and that this ATP level is decreased immediately by enzymatic degradation and/or diffusion. It is generally thought that ATP release levels differ depending on the cell type and experimental conditions, such as temperature (Geyti et al., 2008; Ryu et al., 2010). Given that ATP release shows strong temperature sensitivity, mechanical stimulation at 37°C could induce a greater and more rapid increase in $[IP_3]_i$ in neighboring cells than that at room temperature.

In conclusion, we succeeded in visualizing – for the first time – the intracellular and intercellular wave-like responses of Ins(1,4,5)P_3 to mechanical stimulation. These results revealed that the generation of Ins(1,4,5)P_3 in mechanically stimulated cells is primarily induced through the ATP-independent PLC activation pathway, whereas the ATP-mediated activation of PLC induces the Ca^{2+} response in neighboring cells. However, the mechanism of Ins(1,4,5)P_3 generation in mechanically stimulated cells remains unknown and requires further study.

Materials and Methods

Reagents and media

ATP, ThG, ionomycin, saponin, 1-octanol, and 16-DSA were purchased from Sigma (St Louis, MO). Inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3] was purchased from Alexis (San Diego, CA). Suramin was from Calbiochem (Darmstadt, Germany). U-73122 was from Wako Pure Chemical (Osaka, Japan). All other reagents were of analytical grade.

Modified Hanks' balanced-salt solution buffered with HEPES (HBSS-H) contained 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.81 mM $MgSO_4$, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.6 mM glucose, 4.2 mM $NaHCO_3$, and 20 mM HEPES-NaOH (pH 7.4). Ca^{2+}-free HBSS-H was identical in composition to HBSS-H, except that $CaCl_2$ was omitted and 0.1 mM EGTA was added. Intracellular-like medium (ICM) contained 125 mM KCl, 19 mM NaCl, 10 mM HEPES-KOH (pH 7.3), 1 mM EGTA, and 330 μM $CaCl_2$ (to yield a final concentration of 50 nM free $[Ca^{2+}]$).

Cell culture and transfection

HSY-EA1 cells were cultured for 1 week in a recording chamber consisting of 4×14 mm acrylic-resin cylinders glued to round glass coverslips with silicone rubber adhesive. Cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (Sigma) supplemented with 10% newborn calf serum, 2 mM glutamine, and 100 μg/ml each of penicillin and streptomycin (GIBCO, Carlsbad, CA) as described previously (Moran and Turner, 1993).

Transient transfections were performed using Opti-MEM (GIBCO) containing Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) and 5 μg/ml plasmids (LIBRAvIII or LIBRAvN) according to the manufacturer's protocol.

Mechanical stimulation and reagent application

HSY-EA1 cells were stimulated by poking the cell surface for 0.1 seconds using a glass micropipette (Femtotip; Eppendorf, Hamburg, Germany) controlled with a micromanipulator (InjectMan NI 2, Eppendorf). Fura-2 fluorescence was monitored to examine the intracellular Ca^{2+} mobilization and to verify the integrity of the cell membrane. A vacuum line was placed in the sample chamber and adjusted to maintain a solution volume of ~200 μl. Solution changes were accomplished within 2 seconds by the addition of 1000 μl of fresh solution to the chamber.

Monitoring Ca^{2+} mobilization

HSY-EA1 cells in chambers were washed with HBSS-H and then incubated in the same medium containing 2 μM Fura-2/acetoxymethyl ester (Fura-2/AM, Dojindo Laboratories, Kumamoto, Japan) for 30 minutes at room temperature. After incubation, cells were washed thoroughly with fresh HBSS-H without Fura-2/AM, and kept at room temperature in the dark until use. Images of Fura-2 fluorescence were acquired on a AQUACOSMOS/ASHURA system (Hamamatsu Photonics, Shizuoka, Japan) consisting of a Nikon Eclipse TE300 inverted fluorescence microscope (Tokyo, Japan) equipped with a Nikon S Fluor 40 oil-immersion objective [numerical aperture (NA) 1.3]. Fura-2-loaded cells were excited at 340 and 380 nm, and emission signals were recorded with a cooled 3CCD color camera (Orca 3CCD-CYR; Hamamatsu Photonics), resulting in detection of Fura-2 fluorescence on the Y channel (500-565 nm). Image analysis was performed with AQUACOSMOS 2.6 software (Hamamatsu Photonics). All experiments were performed at room temperature.

Simultaneous monitoring of LIBRAvIII and Fura-2 fluorescence

To simultaneously measure mechanical-stimulation-induced changes in $[IP_3]_i$ and $[Ca^{2+}]_i$ in HSY-EA1 cells, LIBRAvIII- or LIBRAvN-expressing cells were incubated for 2-3 minutes in HBSS-H containing 2 μM Fura-2/AM at room temperature. Simultaneous monitoring of LIBRAvIII or LIBRAvN and Fura-2 was performed with sequential excitation at 380 nm (for Fura-2) and 430 nm (for LIBRAvIII and LIBRAvN). Dual-emission fluorescence signals were acquired on the AQUACOSMOS/ASHURA system, resulting in detection of the CFP signal by the C channel (420-500 nm, for LIBRAvIII and LIBRAvN) and the Fura-2 or YFP signal by the Y channel (for LIBRAvIII, LIBRAvN and Fura-2). To minimize noise, four consecutive fluorescent images of LIBRAvIII at each emission wavelength (420-500 and 500-565 nm) were averaged using the rolling-average method, and used to create relative ratio images (C/Y channels). To visualize Ins(1,4,5)P_3 responses, ratio images (R) were normalized by the ratio image before mechanical stimulation (R_0). Data were analyzed with AQUACOSMOS 2.6 software (Hamamatsu Photonics). All experiments were performed at room temperature.

Estimation of $[IP_3]_i$ in individual cells

$[IP_3]_i$ in individual cells was calculated by:

$$[IP_3]_i = K_d[\Delta R(\Delta R_{max} - \Delta R)^{-1}]^{1/n}, \quad (1)$$

as described previously (Tanimura et al., 2009), where ΔR is $R–R_{min}$, ΔR_{max} is $(R_{max}'–R_{min}')\,R_{min}\,R_{min}'^{-1}$, K_d for LIBRAvIII is 491.5 nM, and n is the Hill coefficient (1.13). Emission ratios in the absence (R_{min}') and presence (R_{max}') of saturating concentrations of Ins(1,4,5)P_3 (20 μM) were monitored after permeabilization by 0.02% saponin (Sigma) in ICM. R_{min} was calculated by:

$$R_{min} = R_{rest} - E_{rest}\,\Delta R_{max}, \quad (2)$$

where R_{rest} is the emission ratio in a resting cell, and E_{rest} is the E (%max) of resting $[IP_3]_i$. The E_{rest} of LIBRAvIII is 0.025, under the assumption that the resting $[IP_3]_i$ of HSY-EA1 cells is 18.16 nM (Tanimura et al., 2009).

Fluorescence recovery after photobleaching (FRAP) analysis

FRAP analysis employed a confocal microscope system (Radiance 2100; Carl Zeiss, Oberkoche, Germany) attached to an inverted microscope (TE 2000; Nikon, Tokyo, Japan) equipped with Nikon Plan Apo 60× H oil-immersion objective (NA 1.4). Fluorescence of the YFP moiety of LIBRAvIII was visualized with a 514-nm excitation beam (10% of maximal power). Fluorescence images were captured using a 560-nm long-pass filter. To bleach fluorescence in the region of interest, the laser intensity was set to maximal power.

Statistical analysis

All results shown are means ± s.e.m. for three or more independent experiments. *P*-values <0.05 (Dunnett's multiple comparison test) represent statistically significant differences.

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