

## EXPANDED ABSTRACT

# Monitoring of IP<sub>3</sub> dynamics during the mechanical stimulation-induced intra- and intercellular Ca<sup>2+</sup> waves in HSY human parotid cell line

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## INTRODUCTION

It is well known that mechanical stimulation elicits Ca<sup>2+</sup> responses in various cell types. Touch stimulation on a single cell induces an increase in the cytosolic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>), which subsequently propagates to neighboring cells as an intercellular Ca<sup>2+</sup> wave. In addition to Ca<sup>2+</sup> entry through mechanosensitive channels, this Ca<sup>2+</sup> response is thought to be induced, at least in part, by inositol 1,4,5-trisphosphate (IP<sub>3</sub>)-dependent Ca<sup>2+</sup> release from intracellular Ca<sup>2+</sup> stores (1-3). However, this hypothesis has not been tested directly due to a lack of the quantitative method to measure the cytosolic concentration of IP<sub>3</sub> ([IP<sub>3</sub>]<sub>i</sub>) in a single living cell.

Recently, we developed a FRET-based IP<sub>3</sub> biosensor, LIBRAvIII, and a method to measure [IP<sub>3</sub>]<sub>i</sub> quantitatively in the single living cell (4). In order to examine the involvement of IP<sub>3</sub> in mechanical stimulation-induced Ca<sup>2+</sup> responses, we monitored IP<sub>3</sub> and Ca<sup>2+</sup> responses simultaneously in HSY-EA1 cells, a human parotid cell line, using LIBRAvIII and the Ca<sup>2+</sup> indicator fura-2.

## METHODS

HSY-EA1 cells were cultured for 1 week in a

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recording chamber. LIBRAvIII and LIBRAvN, an IP<sub>3</sub> insensitive variant of LIBRAvIII (4), were transiently expressed using Lipofectamine 2000. These HSY-EA1 cells were incubated with 2 μM fura-2/AM.

HSY-EA1 cells were stimulated by poking (0.1 sec) the cell membrane surface using a glass micropipette equipped with a micromanipulator (Fig. 1a). Simultaneous monitoring of LIBRAvIII, 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 was acquired on an AQUACOSMOS/ASHURA imaging system, and fluorescence signals were recorded by a cooled 3CCD color camera [CFP signal was detected using the C channel (420-500 nm), and fura-2 or Venus signals using the Y channel (500-565 nm)]. The [IP<sub>3</sub>]<sub>i</sub> in individual cells was estimated as described (4).

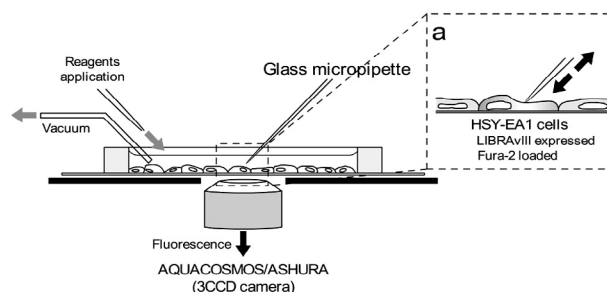


Fig. 1 Measurement of mechanical stimulation-induced IP<sub>3</sub> and Ca<sup>2+</sup> responses in HSY-EA1 cells.

Illustration of the system used for mechanical stimulation and measurement of LIBRAvIII and fura-2 fluorescence. Cells were poked by a glass micropipette as shown in (a).

RESULTS AND DISCUSSION

When a HSY-EA1 cell was poked using a glass micropipette, the Ca<sup>2+</sup> response initiated at the stimulated region and then spread throughout the cell. Subsequently, the Ca<sup>2+</sup> response propagated to neighboring cells as an intercellular Ca<sup>2+</sup> wave. In order to examine the mechanisms by which mechanical stimulation induced Ca<sup>2+</sup> responses, the cells were stimulated in various experimental conditions. Mechanical stimulation-induced Ca<sup>2+</sup> responses were observed in both stimulated and neighboring cells in the absence of extracellular Ca<sup>2+</sup>. Pretreatment of cells with 10 μM U-73122, a phospholipase C (PLC) inhibitor, completely attenuated the Ca<sup>2+</sup> response in neighboring cells. These results indicate that Ca<sup>2+</sup> responses in neighboring cells are primarily mediated by the Ca<sup>2+</sup> release from intracellular stores. In contrast, U-73122 treatment reduced Ca<sup>2+</sup> responses in mechanically stimulated cells by ~ 50% in the presence of extracellular Ca<sup>2+</sup>, but the responses were attenuated completely in its absence. These results indicate that the Ca<sup>2+</sup> response in mechanically stimulated cells results from Ca<sup>2+</sup> entry and PLC-mediated Ca<sup>2+</sup> release.

It has been reported that ATP is involved in the propagation of intercellular Ca<sup>2+</sup> waves after mechanical stimulation (1, 5, 6). Pretreatment of cells with 100 μM suramin, a purinergic receptor blocker, inhibited Ca<sup>2+</sup> responses in neighboring cells almost completely. Although gap junctions have been shown to contribute to intercellular Ca<sup>2+</sup> waves in some cell types (7, 8), the gap junction inhibitors 1-octanol and 16-DSA had no effect on Ca<sup>2+</sup> responses in HSY-EA1 cells. Taken together, these data indicate that mechanical stimulation-induced intercellular Ca<sup>2+</sup> waves in HSY-EA1 cells primarily result from ATP released by stimulated cells (Fig. 2).

In contrast to neighboring cells, suramin reduced Ca<sup>2+</sup> responses in mechanically stimulated cells by only ~ 50%. These suramin resistant Ca<sup>2+</sup> responses were observed even in the absence of extracellular Ca<sup>2+</sup>. One might assume that this Ca<sup>2+</sup> release may be attributable to the disruption of intracellular Ca<sup>2+</sup> stores by mechanical stimulation. However, Ca<sup>2+</sup> release in mechanically stimulated cells was completely inhibited by U-73122. These results suggest that Ca<sup>2+</sup> release in mechanically stimulated cells is induced, at least in part, by an ATP-independent mechanism (Fig. 2).

Our studies indicate that mechanical stimulation-induced Ca<sup>2+</sup> release is mediated by ATP-dependent

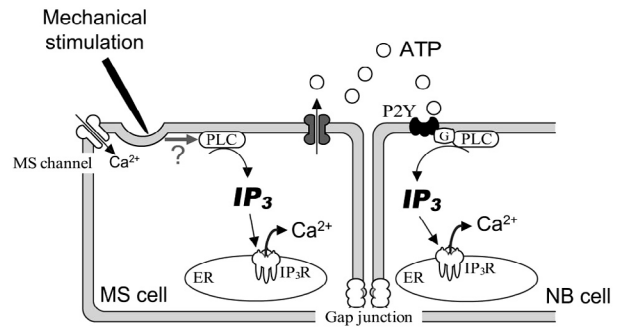


Fig. 2 Mechanical stimulation induces IP<sub>3</sub> generation via ATP-dependent and ATP-independent PLC activation in stimulated and neighboring cells. ER: endoplasmic reticulum. IP<sub>3</sub>R: IP<sub>3</sub> receptor. MS cell: mechanically stimulated cell. NB cell: neighboring cell.

and/or ATP-independent activation of PLC. Therefore, IP<sub>3</sub> generation would be expected to contribute to this process. In order to examine this idea directly, we monitored changes in [IP<sub>3</sub>]<sub>i</sub> during mechanical stimulation-induced Ca<sup>2+</sup> responses in HSY-EA1 cells using LIBRAvIII and fura-2. Mechanical stimulation induced a transient increase in the emission ratio of LIBRAvIII in both stimulated and neighboring cells, and these responses correlated with the Ca<sup>2+</sup> response. In the presence of 10 μM U-73122, the mechanical stimulation-induced increases in the emission ratio of LIBRAvIII in both stimulated and neighboring cells were completely blocked. In addition, mechanical stimulation did not change the emission ratio of LIBRAvN, an IP<sub>3</sub> insensitive variant of LIBRAvIII, even though large Ca<sup>2+</sup> responses were observed. These experiments indicate that mechanical stimulation-induced changes in the emission ratio of LIBRAvIII indeed reflect changes in [IP<sub>3</sub>]<sub>i</sub>.

We then examined the effect of suramin on mechanical stimulation-induced increases in [IP<sub>3</sub>]<sub>i</sub> in HSY-EA1 cells. In the presence of 100 μM suramin, mechanical stimulation-induced increases in [IP<sub>3</sub>]<sub>i</sub> in neighboring cells were completely attenuated. In contrast, suramin had only a small effect on the increase in [IP<sub>3</sub>]<sub>i</sub> in mechanically stimulated cells. These results indicate that mechanical stimulation-induced generation of IP<sub>3</sub> in stimulated cells involves an ATP-independent PLC activation pathway. In neighboring cells, however, IP<sub>3</sub> generation and Ca<sup>2+</sup> responses were mediated by ATP released from stimulated cells (Fig. 2). Given that [IP<sub>3</sub>]<sub>i</sub> was modulated by released ATP, we postulated that ATP may have autocrine effects on mechanical stimulation-induced IP<sub>3</sub> generation. Nevertheless, our data

indicate that the contribution of ATP-mediated pathways in mechanically stimulated cells is very small.

## CONCLUSION

In this study, we show that IP<sub>3</sub>-induced Ca<sup>2+</sup> release contributes to mechanical stimulation-induced Ca<sup>2+</sup> responses in both stimulated and neighboring cells. Our results suggest that Ca<sup>2+</sup> release in mechanically stimulated cells is primarily induced by an ATP-independent pathway, whereas Ca<sup>2+</sup> responses in neighboring cells are mediated by ATP released from stimulated cells. Furthermore, we demonstrate directly that mechanical stimulation induces an increase in [IP<sub>3</sub>]<sub>i</sub> in both stimulated and neighboring cells during Ca<sup>2+</sup> responses. The mechanism of IP<sub>3</sub> generation in mechanically stimulated cells remains unknown, and further experiments will be required to elucidate these pathways.

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