Cell Calcium 47 (2010) 253–263

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Spatio-temporal PLC activation in parallel with intracellular Ca^{2+} wave propagation in mechanically stimulated single MDCK cells

Akira Tsukamoto[a,e], Yasunori Hayashida[b], Katsuko S. Furukawa[b,c,d], Takashi Ushida[a,b,c,d,*]

[a] Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[b] Department of Mechanical Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
[c] Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
[d] Center for Nanobio Integration, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
[e] Translational System Biology and Medicine Initiative (TSBMI), The University of Tokyo, Japan

ARTICLE INFO

Article history:
Received 8 July 2009
Received in revised form
10 December 2009
Accepted 17 December 2009
Available online 8 January 2010

Keywords:
Mechanical stimulation
Phospholipase C (PLC)
Intracellular Ca^{2+} wave
Real-time dual imaging
Subcellular imaging
MDCK

ABSTRACT

Intracellular Ca^{2+} transients are evoked either by the opening of Ca^{2+} channels on the plasma membrane or by phospholipase C (PLC) activation resulting in IP_3 production. Ca^{2+} wave propagation is known to occur in mechanically stimulated cells; however, it remains uncertain whether and how PLC activation is involved in intracellular Ca^{2+} wave propagation in mechanically stimulated cells. To answer these questions, it is indispensable to clarify the spatio-temporal relations between intracellular Ca^{2+} wave propagation and PLC activation. Thus, we visualized both cytosolic Ca^{2+} and PLC activation using a real-time dual-imaging system in individual Mardin–Darby Canine Kidney (MDCK) cells. This system allowed us to simultaneously observe intracellular Ca^{2+} wave propagation and PLC activation in a spatio-temporal manner in a single mechanically stimulated MDCK cell. The results showed that PLC was activated not only in the mechanically stimulated region but also in other subcellular regions in parallel with intracellular Ca^{2+} wave propagation. These results support a model in which PLC is involved in Ca^{2+} signaling amplification in mechanically stimulated cells.

1. Introduction

A mechanical stimulation evoke Ca^{2+} signaling by which cells realize several types of homeostasis, including cell polarity, protein production and membrane trafficking [1–3]. Among several regulators of Ca^{2+} signaling, PLC has been suggested to be involved in mechanically stimulated Ca^{2+} signaling in several cell types [4,5]. For example, inhibition experiments have revealed that PLC inhibitors suppress Ca^{2+} signaling [6–12]. Other studies have shown that IP_3 synthesis following PLC activation is enhanced by mechanical stimulation [13,14]. Although PLC seems to be involved in mechanically stimulated Ca^{2+} signaling, it remains elusive how PLC regulates Ca^{2+} signaling in mechanically stimulated cells.

There are two models for explaining how PLC regulates Ca^{2+} signaling [15,16]. In one model, PLC regulates Ca^{2+} signaling initiation. In this "Ca^{2+} signaling initiation model", extracellular stimulations activate PLC, and then PLC further induces an initial Ca^{2+} increase. In the other model, PLC regulates Ca^{2+} signaling amplification. In this "Ca^{2+} signaling amplification model", PLC amplifies Ca^{2+} signaling by the mechanism in which Ca^{2+} activates PLC which in turn stimulates further Ca^{2+} increase. Thus, in mechanically stimulated cells, if PLC is involved in mechanically stimulated Ca^{2+} signaling, PLC is also supposed to regulate Ca^{2+} signaling in Ca^{2+} signaling initiation and/or in Ca^{2+} signaling amplification.

In order to understand how PLC regulates Ca^{2+} signaling in mechanically stimulated cells, we measured PLC activation dynamics along with Ca^{2+} signaling with a direct method, i.e., real-time dual imaging [17–19]. Real-time dual imaging is a minimally invasive technique that reveals the spatio-temporal interaction of multiple signals without inhibiting them. With this technique, it is possible to observe the spatio-temporal distribution of PLC along with mechanically stimulated Ca^{2+} signaling. If PLC was involved in Ca^{2+} signaling initiation, PLC would be activated in parallel with the initial Ca^{2+} increase. Otherwise, if PLC was involved in Ca^{2+} signaling amplification, PLC would be activated in parallel with the Ca^{2+} increase acceleration, e.g., Ca^{2+} spike or Ca^{2+} wave [17].

Intracellular Ca^{2+} wave is one example of Ca^{2+} signaling that is induced by mechanical stimulation [8,20–22]. Intracellular Ca^{2+} wave is initiated by a subcellular increase in Ca^{2+} (intracellular Ca^{2+} wave initiation) and propagates throughout the cytoplasm (intracellular Ca^{2+} wave propagation) [15,16,23]. Intracellular Ca^{2+} waves have a pronounced feature—i.e., their initiation and propagation are

* Corresponding author. Fax: +81 3 5800 9199.
E-mail address: ushida@m.u-tokyo.ac.jp (T. Ushida).

0143-4160/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ceca.2009.12.008

distinguished in a spatio-temporal manner even within individual cells because of the limited initial Ca^{2+} increase. Therefore, it would be of use to examine whether PLC activation occurs in parallel with two Ca^{2+} signaling features—intracellular Ca^{2+} wave initiation and propagation—in mechanically stimulated intracellular Ca^{2+} waves.

In this study, whether and how PLC activation is involved in mechanically stimulated Ca^{2+} signaling was investigated in MDCK cells. In order to distinguish between Ca^{2+} signaling initiation and amplification in a single mechanically stimulated cell, an intracellular Ca^{2+} wave was induced with subcellular mechanical stimulation. Because subcellular mechanically stimulation was applied in a limited region of plasma membrane, Ca^{2+} signaling initiation in intracellular Ca^{2+} wave—intracellular Ca^{2+} wave initiation—and Ca^{2+} signaling propagation in intracellular Ca^{2+} wave—intracellular Ca^{2+} wave propagation—were expected to be separated. By detecting the region of the plasma membrane that was micro-deformed by subcellular mechanical stimulation with fluorescence decrease, two Ca^{2+} signaling features—intracellular Ca^{2+} wave initiation and propagation—could be distinguished in each single cell. Whether PLC activation was involved in mechanically stimulated Ca^{2+} signaling was investigated with inhibition experiments. How PLC activation was involved in mechanically stimulated Ca^{2+} signaling was further investigated by observing the spatio-temporal distribution of PLC activation along with intracellular Ca^{2+} wave propagation using a real-time dual-imaging system.

2. Materials and methods

2.1. Cell culture

MDCK cells were provided by the RIKEN CELL BANK (Tsukuba, Japan). Cells were cultured in MEM-Eagle (Sigma) supplemented with 10% fetal bovine serum (FBS). Cells were maintained in a culture incubator at 37 °C in a humidified atmosphere of 5% CO_2 and were subcultured every 4–6 days in order to prevent overconfluence. For experiments, cells were detached by treatment with 0.025% trypsin and were seeded onto cover glasses (Matsunami, Japan) at a density of 1×10^4 cells/cm^2. Either for inhibition experiments or for real-time dual imaging of PLC activation and Ca^{2+} increase, cells were cultured for 1–2 days on cover glasses before further procedures.

2.2. Subcellular mechanical stimulation

Subcellular mechanical stimulation was induced by micro-deforming the plasma membrane with micropipettes [24]. To prevent rupture of the plasma membrane, the tips of the micropipettes were heat-blunted so that their diameters were approximately 2–3 μm. Each micropipette was mounted on a manual micromanipulator (Leica, Germany) and was first manipulated so that the tip was close to the top of the plasma membrane of a cultured cell. The plasma membrane above the nucleus was chosen for subcellular mechanical stimulation in order to realize stable stimulations. Prior to stimulation, the tip of the micropipette was softly attached to the plasma membrane surface and then further indented. Micropipette indention depth was approximately 1–2 μm, which was estimated using the scale on the manual micromanipulator.

The region of the plasma membrane that was micro-deformed by subcellular mechanical stimulation was quantified by analyzing the decrease in fluorescence under micro-deformation. This fluorescence decrease was transient and was independent of the fluorescence decrease due to plasma membrane rupture. The percentage of the whole plasma membrane area in which the fluorescence was decreased was quantified with a fluorescence image that was taken just under time of subcellular mechanical stimulation. The time course of the transient fluorescence decrease was also obtained by evaluating the difference in fluorescence between the region with decreased fluorescence and the other plasma membrane regions.

2.3. Inhibition experiments

The ability of Ca^{2+}-signaling inhibitors to inhibit the Ca^{2+} signaling induced by subcellular mechanical stimulation was evaluated by measuring the levels of Ca^{2+} increase. The Ca^{2+} increase was visualized by loading Fura-2AM on MDCK cells. Cells were washed with HEPES buffered saline solution (HBSS (in mM): NaCl 130, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.8, glucose 5.5, HEPES 20; pH adjusted to 7.4 with NaOH) prior to Fura-2AM loading to eliminate FBS. Cells were loaded with Fura-2AM (1 μM in HBSS; Dojindo, Japan) for 20 min at 37 °C. After loading Fura-2AM, cells were washed more than twice with HBSS and were further incubated for 20 min at room temperature for Fura-2AM digestion in cytosol. Inhibitors including thapsigargin (1 μM in HBSS; Sigma), U73122 (1 μM in HBSS; Calbiochem) and neomycin (5 mM in HBSS; Sigma) were loaded on MDCK cells following Fura-2 loading. Inhibitors were loaded for 5 min for thapsigargin; and 30 min for U73122 and neomycin.

For depleting extracellular Ca^{2+} in HBSS, HBSS was exchanged with HBSS containing low Ca^{2+} high Mg^{2+} (NaCl 130, KCl 5.4, $MgCl_2$ 2.6, glucose 5.5, HEPES 20, EGTA 0.1; in mM). Following Fura-2 loading, HBSS was exchanged for low Ca^{2+} high Mg^{2+} HBSS. Low Ca^{2+} high Mg^{2+} HBSS contained extra Mg^{2+} (2.6 mM) because the plasma membrane was shown to fluctuate in a previous study under low Ca^{2+} HBSS without Mg^{2+} [8].

To visualize the increase in Ca^{2+}, cells containing Fura-2 were alternately exposed to fluorescence of two wavelengths, 340 nm and 380 nm, with a high-speed excitation wavelength switcher (Hamamatsu Photonics, Japan). Excitation and emission were transmitted through an appropriate dichroic mirror for Fura-2 imaging (Semrock, FURA-2B) and an oil immersion objective (FLUAR 100×; Zeiss). Emission images were obtained with an EM-CCD camera (Hamamatsu Photonics) every 1 s (approximately 500 ms for each emission). The emission ratio (F_{340}/F_{380}) of the obtained images was analyzed with the image analysis software package Aquacosmos (Hamamatsu Photonics).

2.4. Real-time dual imaging of PLC activation and Ca^{2+} increase

For dual imaging of PLC activation and Ca^{2+} increase, cells were first transfected with DNAs encoding PLCδ1PH-CFP (CFP-PH) and PLCδ1PH-YFP (YFP-PH) (kindly provided by Dr. Kees Jalink, The Netherlands Cancer Institute) with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's procedures. CFP-PH and YFP-PH (CFP-PH/YFP-PH) exhibited Förster resonance energy transfer (FRET) when they were bound to the plasma membrane, and FRET ceased when PH domains were translocated to the cytosol following PLC activation [25]. For DNAs to express fluorescent proteins, cells were incubated for an addition 24–36 h. Before real-time dual imaging of PLC activation and Ca^{2+} increase, cells were stained with Fura-2 using the procedures described in Section 2.3.

Cells containing both CFP-PH/YFP-PH and Fura-2 were alternately exposed to fluorescence at two excitation wavelengths, 380 nm and 440 nm, using a high-speed excitation wavelength switcher (Hamamatsu Photonics). Fura-2 and CFP-PH/YFP-PH were designed to be excited at excitation wavelengths of 380 nm and 440 nm, respectively. From the point of view of avoiding emission cross-talk between Fura-2 and CFP-PH/YFP-PH, separated excitation wavelengths were ideal for excitation; however, we