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Intracellular calcium dynamics and expression of P2Y and IP_3 receptors in a cycling G_1-phase cell

Gabriel J. Mchonde[1, 2], Yoh-ichi Satoh[1, 3], Shinji Yasuhira[4], Chihaya Maesawa[4], Tomoyuki Saino[1]

Department of [1]Anatomy (Cell Biology), [3]Medical Education, and [4]Tumor Biology, Institute of Biomedical Sciences, Iwate Medical University, Yahaba, Iwate, Japan and [2]Department of Biomedical Sciences, School of Medicine and Dentistry, College of Health Sciences, University of Dodoma, Dodoma, Tanzania.

Summary

The regulation of intracellular events is of critical importance in proliferating cells. These events may be altered by signaling molecules linked through cell-cycle regulatory mechanisms. Recent advances have linked the calcium ion (Ca^{2+}) with the progression of the cell cycle through interphase and the different phases of mitosis. However, there has been little explanation on the fundamental relationship of calcium signals and their associated receptors with the interphase subphases. In the present study, to clarify this possible relationship, we investigated how calcium signaling and its associated purinergic receptors are related to the cell cycle between the nucleoplasm and cytoplasm in cultured G_1-interphase cells of *HeLa. S-Fucci2* and fucci/mouse fibroblasts. Ratiometric fluorescence and reverse transcriptase polymerase chain reaction (RT-PCR) techniques were employed to assess the intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) and the expression of purinergic and inositol trisphosphate receptors, respectively. The results obtained revealed the existence of two distinct subcellular increases in $[Ca^{2+}]_i$ in a single individual G_1-phase cell, suggesting variations between the early and late G_1-phases of the cell cycle. In addition to the Ca^{2+} wave, the RT-PCR results indicated variability in the purinergic receptors and inositol 1,4,5-trisphosphate receptor subtypes within G_1-phase cells. Based on these results, we propose that receptor expression and calcium signals are functionally distinct within individual interphase subphases.

Keywords

G_1-interphase cell, calcium signaling, receptor expression, cell cycle

Introduction

Intracellular signaling depends on the expression of receptors, the functional activities of these receptors, and the responses following stimulation or activation by either external or internal stimuli. Specific receptor genes located within the nucleus are transcribed and then translated to synthesize a specific protein, which is then utilized to make a receptor. These receptors may function within the nucleus itself or be exported to the cytoplasm or plasma membrane, allowing gene expression. The process of receptor gene transcription has been reported to involve several intracellular signaling molecules, such as calcium ion (Ca^{2+}), which are also key players in the cell cycle (Dubyak, 1991). Cell proliferation involves repeated intracellular events arranged in a cyclic manner, termed the cell cycle (interphase-mitosis-cytokinesis-interphase), which result in the formation of new cells (Takuwa et al., 1993). A number of molecular mechanisms have been proposed to account for cell proliferation that involve several intracellular signaling molecules, including Ca^{2+}, cyclic adenosine monophosphate, hormones, and growth factors.

Ca^{2+} is a universal signaling molecule involved in the cell cycle stages and the progression through various intracellular signaling cascades, playing a major role as a second messenger or agent for the phosphorylation processes. The mobilization of Ca^{2+} from intracellular stores and the extracellular matrix has previously been associated with numerous activities that occur during cellular proliferation, such as the expression and activation of early genes

*To whom all correspondence should be addressed.
Gabriel J. Mchonde, DVM. and Tomoyuki Saino, MD., Ph.D.
Department of Anatomy (Cell Biology), Iwate Medical University,
2-1-1 Nishitokuta, Yahaba-cho, Shiwa-gun, Iwate 028-3694, Japan
Tel.: +81-19-651-5111, Fax: +81-19-908-8006
E-mail: gmchonde@yahoo.co.uk, mchonde@iwate-med.ac.jp
E-mail: tsaino@iwate-med.ac.jp

involved in the transition from a resting cell (G_0) to G_1-phase, retinoblastoma protein (Rb) phosphorylation in G_1-phase, and the activation of cyclin-dependent kinases (Cook and Lockyer, 2006; Dolmetsch, 2003; Parkash and Asotra, 2010; Tyson and Novak, 2008). This process involves a universal mechanism that follows the binding of a hormone or growth factor to G-protein coupled receptors (GPCRs), primarily Gq/11 subtypes, or tyrosine kinase receptors leading to the activation of phospholipase C (PLC) and PLCγ, respectively. PLC then cleaves phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP_3), and diacylglycerol. The binding of IP_3 to IP_3 receptor (IP_3Rs) channels present in intracellular Ca^{2+} stores, such as the endoplasmic reticulum, causes the efflux of Ca^{2+} from these stores and increases the intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$). The presence of Ca^{2+} in the cytosol then activates another Ca^{2+} channel, the ryanodine receptor, resulting in further Ca^{2+} release through a process known as Ca^{2+}-induced Ca^{2+} release (Berridge, 2009; Resende et al., 2013). As these stores become depleted, Ca^{2+} entry from the extracellular matrix occurs via store-operated Ca^{2+} entry (SOCE) mechanisms in order to refill the depleted stores (Putney, 2005).

Transmembrane metabotropic GPCR-purinergic receptors (P2YRs) are activated by extracellular nucleotides (adenosine diphosphate, adenosine triphosphate, uridine diphosphate, and uridine triphosphate) and ensure rapid intracellular signaling (Zimmermann, 2016). Extracellular adenosine triphosphate (ATP) is known to exert specific activities that regulate a number of cellular functions, such as development, proliferation, and differentiation (Abbrachio and Burnstock, 1998), by stimulating P2YRs. The binding of ATP to P2YRs has been associated with intracellular Ca^{2+} signaling pathways, leading to alterations in $[Ca^{2+}]_i$ dynamics through the IP_3 pathway (Dubyak, 1991; Kamada et al., 2012; Moriguchi-Mori et al., 2016; Saino et al., 2002) and has previously been linked with cell proliferation (Cullen and Lockyer, 2002; Ding et al., 2010; Minaguchi et al., 2006; Rey et al.,2010; Zhong et al., 2010).

Intracellular (nuclear and cytoplasmic) and surface (extracellular) receptors, including P2YRs, depend on the expression of various genes that are transcribed as a message by mRNA for a particular receptor protein. The receptor genetic code is stored in DNA and interpreted by gene expression, which is often expressed by the synthesis of receptor proteins. Cell cycle control mechanisms involve several types of gene regulation processes that give the cell control over its structures and functions (Andrade et al., 2011; Benito et al., 1998; Moreno and Nurse, 1994; Schwob et al., 1994; Tyson and Novak, 2008), hence its versatility and adaptability during cycle progression in various stages (G_1-phase, S-phase, G_2-phase, and Mitosis-phase); as a result, the up- or down-regulation of certain receptors or their sensitivity occurs in response to biological stimulants.

Although the question of how Ca^{2+} signaling affects the cell cycle in general has been extensively explored, few studies have addressed the expression and properties of the Ca^{2+} signaling in certain cell cycle stages (Manzoli et al., 2004; Poenie et al., 1986; Ratan et al., 1986; Russa et al., 2009; Steinhardt and Alderton, 1988; Volpi and Berlin, 1988; Whitaker, 2006). However, most of these investigations have reported the general findings of Ca^{2+} signaling of the whole cell in certain stages of the cell cycle and not the properties and associated receptors of Ca^{2+} signaling in the subphases of interphase. It is therefore necessary to examine the specific properties and associated receptors of the main players in Ca^{2+} signaling to clarify the differences in signaling among the subphases of interphase. We previously found that nucleoplasmic and cytoplasmic fluctuations in $[Ca^{2+}]_i$ ($[Ca^{2+}]_n$ and $[Ca^{2+}]_c$, respectively) in early-to-late G_1-phase cells differed between and within the interphase subphases (Mchonde et al., 2015) following a stimulation with extracellular ATP. These findings raised concerns as to whether the variations observed are based on cellular activities and/or receptor expression in a particular interphase sub phase.

Therefore, in this study, to determine the properties and characteristic expression of P2YRs and IP_3Rs in the G_1-stage of an interphase cell, we investigated the effects of ATP on the increase in $[Ca^{2+}]_i$ in G_1-interphase using a real-time confocal microscope. We aimed to decode the expression of P2YR and IP_3R in relation to the characteristics of receptor-mediated $[Ca^{2+}]_i$ dynamics in a G_1-interphase cell. To accomplish this objective, *HeLa.S-Fucci2* and fMFB cells, non-excitable cell lines, were used. We compared both synchronized and unsynchronized cells using calcium imaging and reverse transcriptase polymerase chain reaction (RT-PCR) techniques.

Materials and methods

Cell lines and culture condition

Primary fibroblast cell culture

All animal experiments were conducted in accordance with the Iwate Medical University Institutional Animal Care and Use Committee. Infant fucci mice (P21) were obtained as a gift from Prof. Hidemitsu Harada (Department of Regenerative Anatomy, Iwate Medical

University) and used as a source for fMFB cells. The heads and necks of infant mice were sprayed with 70% ethanol, followed by euthanasia by cervical decapitation using scissors. A skull midline incision, posterior to anterior, was made to free the skull from the scalp. The cranium was opened and the flaps carefully removed in order to expose the dura matter. The meninges were removed using fine forceps and placed onto a 10-cm petri dish containing 4 mL of full growth media in Dulbecco's modified Eagle medium (DMEM, Gibco, Life Technologies, Canada), 5% fetal bovine serum (FBS, Gibco), 1% amphotericin B (Gibco), and 1% penicillin-streptomycin (Gibco) without phenol red. The meninges were allowed 5 min to attach to the bottom of the dish, smeared onto the petri dish, and then incubated with 10 mL of pre-warmed DMEM medium supplemented with 5% FBS, 2 mM L-glutamine, 2.5 µg/mL of amphotericin B, and 100 µg/mL penicillin-100 U/mL streptomycin in a humidified incubator with 5% CO_2 at 37 °C. The medium was changed every third day in order to allow for the proliferation of fibroblasts from the explants. After two weeks, yy explants were removed from the dish. The fibroblasts were incubated until confluence, and then the cells were passaged with 0.1% trypsin-EDTA (Gibco) before being split for subsequent experiments and cryopreserved in liquid nitrogen with preservation medium containing full growth media, 30% FBS, and 15% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA) until further experiments.

HeLa.S-Fucci2 cells

HeLa cells expressing fluorescence ubiquitin cell cycle indicator (Fucci) probes (*HeLa.S-Fucci2* [RCB 2867]) were provided by the RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. Cells were maintained in DMEM supplemented with 5% FBS and 1% penicillin-streptomycin in a 5% CO_2 humidified atmosphere at 37 °C. Cells between passages 2 and 8 were used throughout the experiments.

Expression of cell cycle stages by fucci methods

With *HeLa.S-Fucci2* and fMFB cells, the expressions of *Cdt1* and Geminin proteins indicate the various stages of the cell cycle. Previous studies on cell cultures demonstrated that *mCherry-hCdt1(30/120)* (red) signals occur in nuclei in the early G_1-phase, decrease with the transition to the S-phase, and disappear in the S-phase. Likewise, *mVenus-hGeminin (1/110)* (green) signals occur in nuclei in the early S-phase and increase towards the G_2M and M phases of the cell cycle (Sakaue-Sawano et al., 2011). Using these signals, we identified the different stages of the cell cycle as follows: G_1-phase cell nuclei were red, while nuclei transitioning between the G_1- and S-phases were yellow. In the S-, G_2M-, and M-phases, the nuclei were green.

Cell treatments

Cells were sub-cultured on cover slips in full growth media and in a 5% CO_2 at 37 °C for 48-72 h in order to allow for the adherence to the culture plate and rich confluence. Thereafter, cells were grouped into two cohorts: a full growth media (control) cohort, and full growth media cohort containing 3 µM arcyriaflavin-A (a potent Cdk4/cyclin D1 inhibitor; Tocris, Bioscience, Bristol, UK); the cells were incubated for 48 h. Growth media was replaced every 32 h. All cohorts were treated under similar experimental conditions. Arcyriaflavin-A inhibits Cdk4/cyclin D1 complex activity from the phosphorylating Rb, causing it to remain bound to transcription factor E2F (the Rb-E2F complex), which results in cell cycle arrest at the G_1-phase (Baughn et al., 2006; Malumbres et al., 2004; Sanchez-Martinez et al., 2003; Zhu et al., 2003). Thus, arcyriaflavin-A was used to synchronize research cells into the G_1-phase of interphase.

Measurement of free $[Ca^{2+}]_i$ with Indo-1/AM

Adherent cells on coverslips were placed into modified Sykes-Moore chambers. The cells were gently washed twice with Ca^{2+}-free HEPES-buffered Ringer's solution (HR) containing 118 mM NaCl, 5.5 mM D-glucose, MEM amino acid solution (Gibco), 4.7 mM KCl, 1.13 mM $MgCl_2$, 1 mM NaH_2PO_4, 10 mM Hepes-NaOH, 2 mM sodium L-glutamate, 0.2% bovine serum albumin (Sigma), and 0.5 M EGTA. The pH was adjusted to 7.4 with 4 M NaOH. The cells were loaded with the Ca^{2+}-sensitive dye Indo-1/AM (a ratiometric fluorescence dye, 2 µM; Dojindo, Kumamoto, Japan) in HR and incubated in a 5% CO_2 humidified atmosphere at 37 °C for 30 min. Excessive dye was removed by gently washing the cells twice with standard HR.

The loaded cells in perfusion chambers were mounted and observed using a real-time confocal microscope (RCM/Ab, a modified version of Nikon model RCM-8000; Nikon, Tokyo, Japan) with an inverted microscope equipped with an argon-ion laser (TE-300, Nikon). Fluorescence emission was directed into a pinhole diaphragm through an H_2O-immersion objective lens (Nikon C Apo 40×, N.A. 1.15; Nikon). Specimens were exposed to a blue-green fluorescence

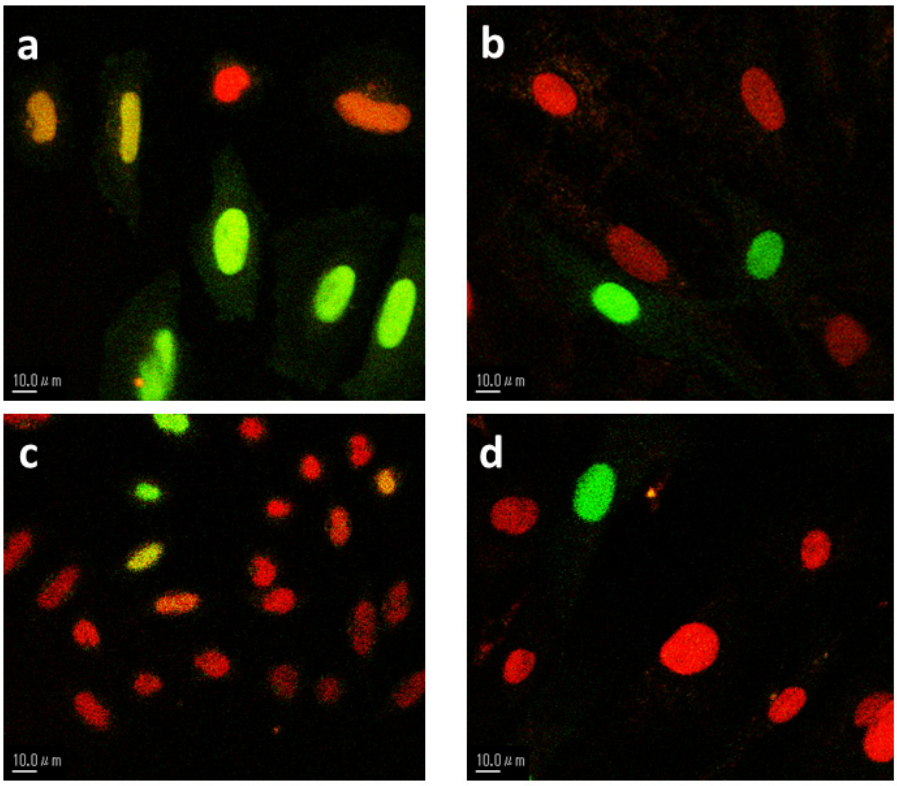


Fig. 1. Confocal micrographs of cultured *HeLa.S-Fucci2* (a and c) and fMFB cells (b and d). Representative images showing fluorescence ubiquitin cell cycle indicator (*fucci*) nuclei characteristics differentiating among various stages of the cell cycle following blue-green (BG: 488 nm) excitation. Red: G_1-phase cell, Yellow: G_1S-phase cell, Green: S/G_2M/M-phase cells. a and b: non-synchronized cohorts; c and d: synchronized cohorts.

(BG; 488 nm) excitation beam in order to establish the cell cycle stage of individual cells; nuclei in the G_1-, G_1S-, and S/G_2/M-phases were red, yellow, and green, respectively. A dichroic mirror splits the emission at 440 nm and directs it into two separate photomultipliers. In this system, the acquisition time per image frame was 1/15 s. In order to obtain a confocal image with maximal spatial resolution, it was necessary for eight frames to be integrated. The image ratios were computed from fluorescence intensities of less than 440 nm ($F_{<440}$) to that greater than 440 nm ($F_{>440}$) with a higher ratio indicative of higher $[Ca^{2+}]_i$. The ratios were displayed in pseudo color with 256 colors, with red representing higher $[Ca^{2+}]_i$ and purple or blue low $[Ca^{2+}]_i$. A preliminary examination with UV excitation revealed that fucci-fluorescence did not interfere with $[Ca^{2+}]_i$. During imaging, the cells were continuously perfused with Ca^{2+}-free HR containing the selected agonists and/or antagonists at a rate of 1 mL per minute. Experiments were conducted at room temperature and at a controlled temperature (Warner Bipolar temperature controller, model CL-100; Warner instruments, Hamden, CT, USA).

Cell Perfusion

$[Ca^{2+}]_i$ changes were examined following continuous perfusion with standard HR containing the following agonists and/or antagonists: adenosine triphosphate (ATP, a purinergic receptor agonist, 10 μM; Kohjin, Tokyo, Japan), uridine triphosphate (UTP, an agonist for $P2Y_{2,4,6}$, 20 μM; Sigma), 2-aminoethyl diphenylborinate (2-APB, an IP_3R antagonist, 100 μM; Tocris Bioscience), U73122 (a phospholipase C inhibitor, 10 μM; Sigma), Suramin (a G-protein antagonist: 100 μM; Sigma), xestospongin C (a selective and membrane permeable inhibitor for IP_3Rs: 1 μM; Sigma), and reactive blue-2 (RB-2, a P2YR antagonist, 100 μM; Sigma).

Determination of P2YR and IP_3R expression by RT-PCR

HeLa.S-Fucci2 and fMFB cells were seeded onto respective cell culture plates on cover-slips in DMEM/F12 with 5% FBS and 1% penicillin-streptomycin. After 72 h, the cells were observed under a confocal

microscope with BG excitation to examine the stages of the cell cycle. We confirmed that cells in the G_1-phase synchronized group had >75% G_1-cells, while those in the non-synchronized (control) group had mixed stages (mostly less than approximately 30% G_1-cells). Total RNA was separately isolated using a commercially available RNeasy Micro Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions for monolayer adherent cells. The RNA levels were measured by spectroscopy at 260 nm. First-strand cDNA synthesis and RT-PCR were performed using ReverTra Ace -α-® (Toyobo, Osaka, Japan) in a thermal cycler (PC-701; ASTEC, Fukuoka, Japan). Previously reported primer sequences were used to amplify P2YR and IP_3R mRNA expression in *HeLa.S-Fucci2* and fMFB cells, as shown in Tables 1 and 2. The thermal cycling protocol was as follows: 94 °C for 1 min, followed by 35 cycles of 94 °C for 45 s, 60 °C for 30 s, and 72 °C for 2 min, with a final extension at 72 °C for 10 min. PCR products were separated by electrophoresis on a 2% agarose gel and visualized with an ethidium bromide gel stain. Images of the gels were obtained using a Polaroid MP4 Land Camera (Polaroid, Minneapolis, MN, USA). Each RT-PCR was repeated for three or more independent experiments.

Results

G_1-phase cell $[Ca^{2+}]_i$ responses to extracellular ATP

First, we checked the ability of cells to express the cell cycle stages following exposure to blue-green fluorescence by identifying individual cells. As shown in Fig. 1, all of the nuclei reflected the cell cycle stages: nuclei in G_1, G_1S, and S/G2M were red, yellow and green, respectively (Fig. 1). We next analyzed the effects of extracellular ATP on the control and synchronized *HeLa.S-Fucci2* and fMFB cohorts. We set the focal plane on the equatorial planes of the cells and placed the regions of interest (ROIs) for the time course of $[Ca^{2+}]_i$ dynamics on these cells. No spontaneous $[Ca^{2+}]_i$ changes were observed in these cells. The experiment generally employed perfusion with 10 μM ATP in the absence of extracellular Ca^{2+}. The exposure of *HeLa.S-Fucci2* and fMFB cells to extracellular ATP increased $[Ca^{2+}]_i$, which was expressed as a transient wave that had a characteristic spike with an apparent peak, followed by a biphasic time course decrease (Fig. 2), indicating rapid and slow decaying times of Ca^{2+} signals in the nucleoplasm and cytoplasm. This result indicates that the intracellular Ca^{2+} responses are mediated by rapid release from nucleoplasmic and cytoplasmic Ca^{2+} stores followed by initial rapid removal by Ca^{2+} pumps (plasma membrane and sarcoendoplasmic reticulum ATPase) and slow but effective intracellular Ca^{2+} buffers within the two cellular compartments. The responses observed varied in cells within the same experiment, as follows: in some cases, the $[Ca^{2+}]_i$ wave commenced in the nucleoplasm or cytoplasm; in others, the wave amplitude was markedly stronger in the nucleoplasm and overlapped with the cytoplasmic responses (experiments, n=21; *HeLa.S-Fucci2* cells: n=11; fMFB cells); in others, the nucleoplasmic wave amplitude was higher than the cytoplasmic wave amplitude but did not overlap the cytoplasmic wave (experiments, n =8; *HeLa.S-Fucci2* cells); and in others, an equal increase was observed between the two cellular compartments (n=9; *HeLa.S-Fucci2* cells: n= 7; fMFB) (Fig. 3).

It has already been established in a number of non-excitable cells, including *HeLa* and fibroblasts, that ATP acting on P2 purinergic receptors, particularly metabotropic P2YRs, elicits the G-protein-mediated activation of PLCβ, which is followed by the formation of IP_3 and release of Ca^{2+} from intracellular stores through IP_3-senstive Ca^{2+} channels. To identify the Gq/11subtypes involved in the mechanisms responsible for these increases in $[Ca^{2+}]_i$, the effects of U73122 (a potent PLC inhibitor) and suramin (an antagonist of G-proteins) were assessed in both the control (Fig. 4) and synchronized cohorts (data not shown) in *HeLa.S-Fucci2* cells. U73122 (10 μM; n = 5) and suramin (50 μM; n = 5) completely blocked ATP-induced increases in $[Ca^{2+}]_i$ (Fig. 4a and b), indicating that PLCβ and G-proteins are involved in the increases in $[Ca^{2+}]_i$.

We also attempted to clarify whether or not IP_3-sensitive Ca^{2+} stores play any role in $[Ca^{2+}]_i$ increases using xestospongin C (a selective and membrane-permeable inhibitor of IP_3Rs), reactive blue-2 (RB-2: a P2YR antagonist), and 2-aminoethyl diphenylborinate (2-APB: an IP_3R inhibitor) (Fig. 4c and d). Xestospongin C (1 μM) strongly blocked ATP-induced $[Ca^{2+}]_i$ increases (n = 8) (Fig. 4c). RB-2 (100 μM) also inhibited $[Ca^{2+}]_i$ elevations induced by 10 μM ATP (Fig. 4d). However, while 2-APB (100 μM) did not completely inhibit the ATP-induced increase in $[Ca^{2+}]_i$ in *HeLa.S-Fucci2* cells, it successfully prevented the increase in fMFB cells (data not shown). This result is consistent with previous findings by Soulsby et al., which showed the variable inhibition responses of 2-APB in various cell types (Soulsby et al., 2002). Collectively, these present and previous findings suggest the universal involvement of the IP_3 pathway system mediated through metabotropic GPCR-purinergic receptors and

the activation of PLCβ, which induces the mobilization of Ca^{2+} from intracellular stores. However, these results did not explain why two different responses were observed in G_1-phase cells. These results prompted us to investigate the expression of receptors involved in the ATP-mediated Ca^{2+} dynamics observed in a G_1-interphase cell using RT-PCR.

Expression of P2YR and IP_3R mRNA in G_1-phase *HeLa.S-Fucci2* cells

In order to identify the receptor isoforms responsible for the calcium signals observed in the G_1-phase, the mRNAs for the P2YR and IP_3Rs isoforms were isolated from *HeLa.S-Fucci2* cells in the synchronized (arcyriaflavin-A treated) and non-synchronized (control) cohorts and analyzed by RT-PCR. Amplified PCR products of the expected sizes for P2YRs were obtained (Table 1) in both cohorts, while β-actin was used as a positive control and molecular standards as a control for the quantity of cDNA loaded onto the gel.

In the control cohort, the expression of P2YRs was weak for $P2Y_1$ and $P2Y_{13}$ mRNAs and completely absent for $P2Y_4$ and $P2Y_{11}$ mRNAs (Fig. 5A). In the synchronized cohorts, the *HeLa.S-Fucci2* cells PCR-amplified products were weak for $P2Y_1$, $P2Y_6$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ mRNA and completely absent for $P2Y_2$ and $P2Y_{11}$ mRNA. However, the presence (appearance) of $P2Y_4$ mRNA was noted (Fig. 5B). Similarly, amplified PCR products of the expected sizes were obtained in *HeLa.S-Fucci2* cells for IP_3Rs (Table 1). In the control cohorts, all three IP_3R isoform mRNAs (types I, II, and III) were expressed, whereas the IP_3R_3 isoform displayed a weak appearance and IP_3R_1 was completely absent in the synchronized (arcyriaflavin-A treated) cohorts (Fig. 5C and D).

Expression of P2YRs and IP_3Rs in G_1-phase fucci/mouse fibroblasts

The P2YR and IP_3R isoforms expressed in G_1-phase fMFB cells with or without pre-treatment with arcyriaflavin-A were concurrently assessed. The mRNA of the P2YR and IP_3R isoforms was isolated from the synchronized (arcyriaflavin-A treated) and non-synchronized (control) cohorts and analyzed by RT-PCR. Amplified PCR products of the expected sizes for P2YRs were obtained (Table 2) in both cohorts, while GADPH was used as a positive control and molecular standards as a control for the quantity of cDNA loaded onto the gel.

In the control cohort, the expression of P2YRs was absent for $P2Y_1$, $P2Y_4$, $P2Y_{10}$, and $P2Y_{13}$ isoform mRNA but present for $P2Y_2$, $P2Y_6$, $P2Y_{12}$, and $P2Y_{14}$ isoform mRNA (Fig. 6A), although the expression of $P2Y_2$ and $P2Y_6$ mRNA was faint. In the synchronized cohorts, the fMFB cell PCR-amplified products were weak for $P2Y_6$ isoform mRNA and completely absent for $P2Y_1$, $P2Y_4$, $P2Y_{10}$, $P2Y_{12}$, and $P2Y_{13}$ mRNA. However, the marked presence (appearance) of $P2Y_2$ and $P2Y_{14}$ isoform mRNA was noted (Fig. 6B). Similarly, amplified PCR products of the expected sizes were obtained in fMFB cells for IP_3Rs (Table 2). In the control cohorts, all three IP_3R mRNAs were expressed, although IP_3R_1 had a faint appearance. However, in the synchronized (arcyriaflavin-A treated) cohorts, the appearance of the IP_3R_1 mRNA isoform was weak, and the IP_3R_2 mRNA isoform was not detected at all (Fig. 6C and D).

Characterization of purinergic receptors by extracellular UTP on intracellular Ca^{2+} mobilization in a G_1-interphase cell

As shown in Fig. 5 and 6, we examined the functional roles of P2YRs in G_1-phase cells. We challenged cells with UTP to induce a $[Ca^{2+}]_i$ response in synchronized and non-synchronized cells in the absence of extracellular Ca^{2+} (Fig. 7a and b) in an attempt to obtain direct evidence for the down-regulated expression of P2YR subtypes on RT-PCR. The response characteristics of G_1-phase cells to 20 μM of extracellular UTP were similar to those observed in ATP-stimulated cells in terms of amplitude characteristics but were weaker than those to ATP in both cohorts. In some experiments, the responses were markedly stronger in the nucleoplasm than in the cytoplasm (n = 4) but were equal between the two cellular compartments (n = 6) in both cohorts in other experiments. However, in the synchronized cohorts, the responses were markedly weaker than those observed in the non-synchronized cohorts (Fig. 7b).

Discussion

The mobilization of Ca^{2+} from internal stores is known to play key roles in cell cycle progression in normal and cancerous tissues. Extracellular nucleotides, such as ATP and UTP, have been shown to stimulate purinoceptors and subsequent Ca^{2+} release from internal stores via the activation of the IP_3 pathway, which leads to calcium signaling in nucleoplasmic and cytoplasmic cellular compartments. Based on the results of the present study, we propose that a relationship exists between and within cell cycle stages and calcium signals, which are dependent on the up- and down-regulation of receptors in each subphase of interphase cells. In this study, we found

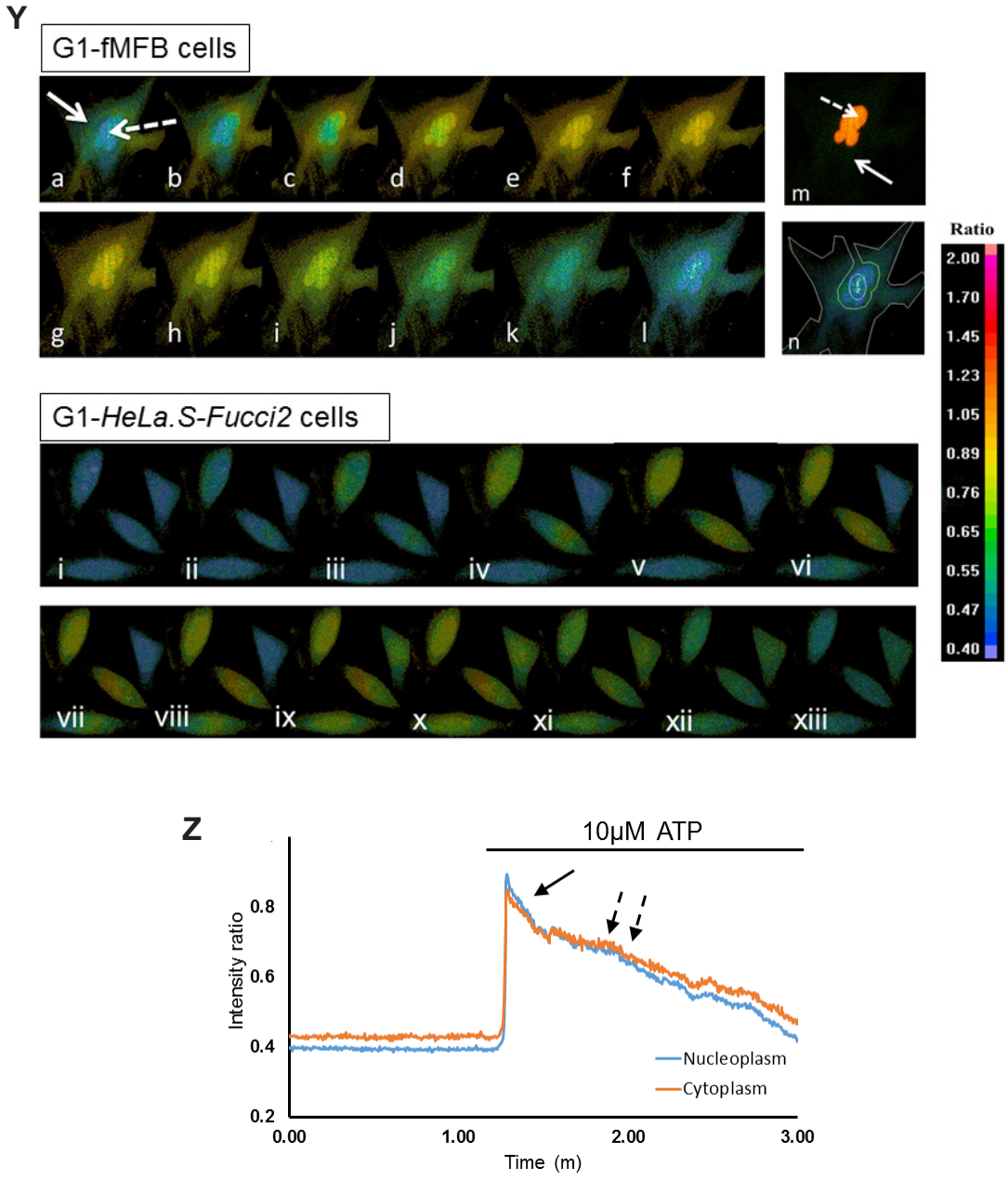
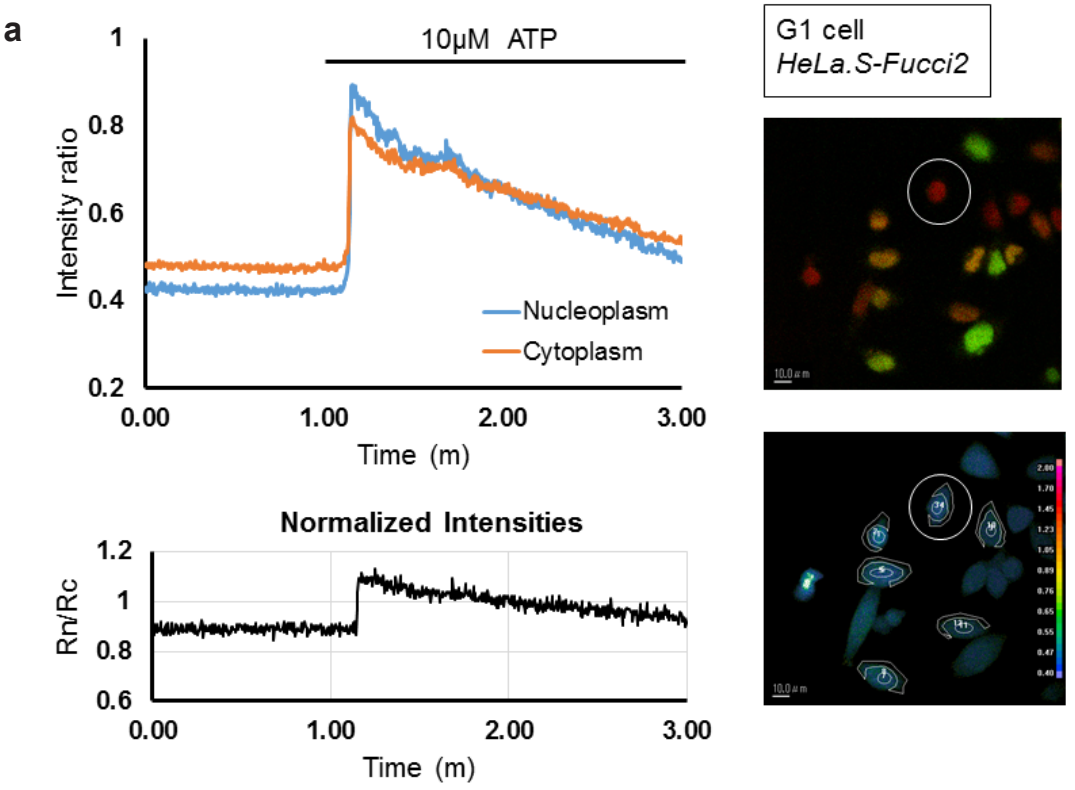


Fig. 2. Fluorescence micrographs showing the representative time course of the $[Ca^{2+}]_i$ changes in an individual G1-phase cell in response to ATP in the absence of extracellular Ca^{2+}. (Y) A single Indo-1AM-loaded G_1-fMFB cell (*a-l*) and G_1-*HeLa.S-Fucci2* cell (*i-xiii*) were challenged with 10 μM ATP in the presence of a 0.5-mM excess of EGTA. Note that the $[Ca^{2+}]_i$ wave dynamics between the cytoplasm (*white bold arrow*) and nucleoplasm (*white dashed arrow*) commenced in the cytoplasm before spreading into the nucleoplasm. However, the intensity of this response was higher in the nucleoplasm than in the cytoplasm. *m*: BG excitation; *n*: UV excitation with a description of how the data were collected. (Z) Representative response amplitudes between the two cellular compartments. The change in the $[Ca^{2+}]_i$ exhibited a biphasic time course for the fast (*black bold arrow*) and slow (*black dotted arrow*) phases of decay. The ratio bar indicates the fluorescence intensities displayed as pseudo color, with red representing higher $[Ca^{2+}]_i$ and purple or blue lower $[Ca^{2+}]_i$.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HeLa.S-Fucci2
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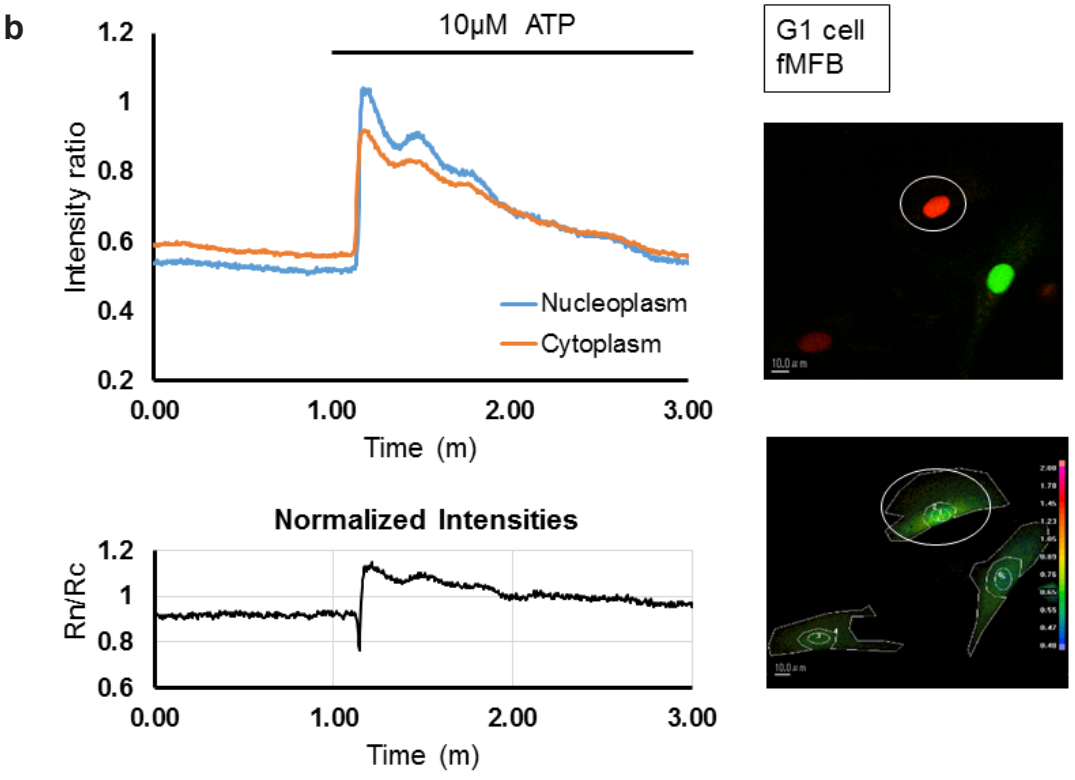
b
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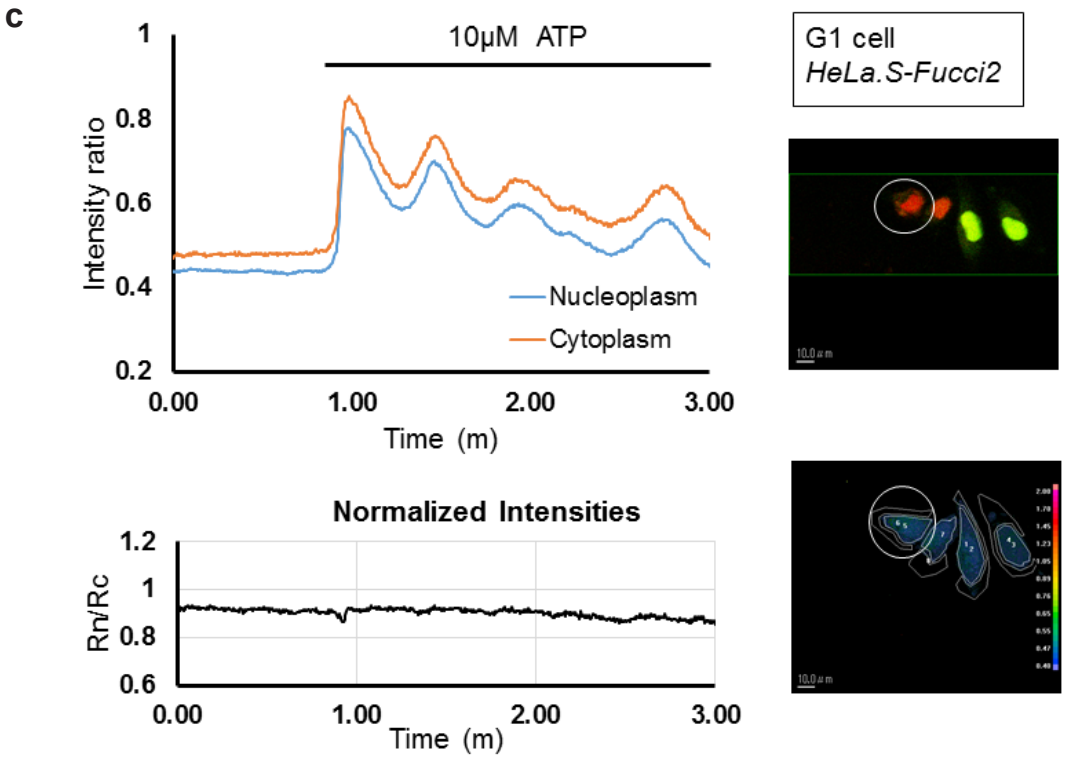

Fig. 3. Variable patterns of the elementary effects of ATP on $[Ca^{2+}]_i$ transients in the G_1-stage of *HeLa.S-Fucci2* and fMFB cells. (a and b) The most common Ca^{2+} release patterns from intracellular stores observed during ATP-induced changes, in which the amplitude of the elementary $[Ca^{2+}]_i$ signals in the nucleoplasm was markedly stronger than that in the cytoplasm: (a) Events in which the wave started in the nucleoplasm and moved to the cytoplasm; (b) Events in which the Ca^{2+} wave started in the cytoplasm and moved to the nucleoplasm. Events in (c) represent ATP-induced responses that had nucleoplasmic amplitudes that did not overlap with those of the cytoplasm. In all of the experiments, the cells were challenged with 10 μM ATP in the presence of a 0.5-mM excess of EGTA. The inserts show photomicrographs of the G1-phase cell, the nucleus of which was cherry red. Rn/Rc: normalized fluorescence ratio at time T, Rn: nucleoplasmic ratio at time T, Rc: cytoplasmic ratio at time T, where R=Ft/Fo. Ft: fluorescence intensity at time T; Fo: fluorescence intensity at time T=0.

that the expression of P2YRs and IP_3Rs was altered and intracellular calcium signals varied within G_1-interphase cells as well as between the nucleoplasm and cytoplasm of the cell.

The cell nucleus is surrounded by a double membrane, the nuclear envelope, which is re-formed during the telophase stage of the cell cycle and separates the nucleoplasm from the cytoplasm throughout interphase (G_1, S, G_2), acting as a semipermeable membrane for controllable communication between the two cellular compartments. Previous studies have demonstrated the existence of a nuclear-cytosolic Ca^{2+} gradient in a number of cell types (Hardingham et al., 1997; Resende et al., 2013; Santella and Kyozuka, 1997; Williams et al., 1987); however, the calcium signals were not examined in relation to the cell cycle between the two cellular compartments. According to Zimmerman, "the major physiological roles of extracellular nucleotides are to act as primary signals in the release of other messenger substances thereby controlling multiple physiological mechanisms" (Zimmermann, 2016). We also previously found that the intracellular Ca^{2+} signal between the nucleoplasm and cytoplasm of an interphase cell varies by the stage of cell division, and these variations are based on the nuclear and cytoplasmic cellular activities during interphase cell cycle stage (Mchonde et al., 2015). The altered Ca^{2+} signal observed in the present study in G_1-phase cells as a response to extracellular ATP may reflect important physiological events during cell cycle progression, such as cell growth, G_1-cell cycle gene expression, and cytoskeleton dynamics, which lead to

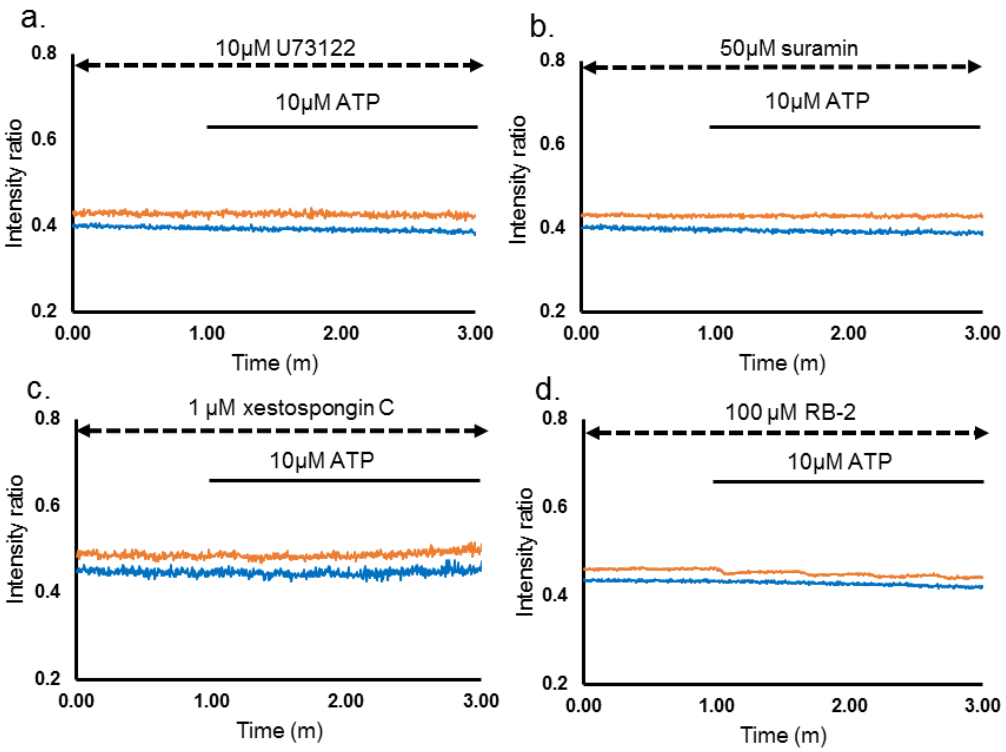

Fig. 4. Effects of IP_3 pathway inhibitors on the mobilization of Ca^{2+} from intracellular stores with extracellular ATP-induced $[Ca^{2+}]$ changes. a: U73122, a potent PLCβ antagonist (10 μM); b: suramin, a G-protein antagonist (50 μM); c: xestospongin C, a selective and membrane-permeable inhibitor for IP_3Rs (1 μM); d: reactive blue (RB-2), a P2YR antagonist (100 μM). Note that all inhibitors successfully inhibited Ca^{2+} release from intracellular stores. Skyblue line: nucleoplasmic ratio, orange line: cytoplasmic ratio.

the differentiation of these cells between the early and late G_1-stages of interphase.

Various cellular activities during resting and active states, including cell cycle events, require energy to achieve the desired effects. This energy is released by the intracellular breakdown of ATP molecules generated or stored within the cell, for which Ca^{2+} is an important secondary messenger and phosphorylation agent. During the early G_1-phase, the newly formed cell exhibits higher levels of physiological activities, such as cell growth and cytoskeletal activities (Abbrachio and Burnstock, 1998; Tyson and Novak, 2008), which require more energy to be effective. These energy-dependent activities may be responsible for strengthened Ca^{2+} signaling in order to facilitate agonist-induced ATP production. Such activities are in contrast to those in late G_1-cells, which are fully matured, resting, and preparing for the next stage of the cell cycle. This hypothesis may therefore further explain variations in the Ca^{2+} signal observed in G_1-interphase cells.

The stimulation of membrane P2YRs has been linked to the agonist-induced release of ATP (Elsing et al., 2012), in which the extracellular stimulation of GPCRs (Gαq) increases $[Ca^{2+}]_i$ via the IP_3 pathway, which in turn directly results in agonist-induced ATP release via exocytosis (De Ita et al., 2016; Gruenhagen and Yeung, 2004; Liu et al., 2005; Osipchuk and Cahalan, 1992; Praetorius and Leipziger, 2009). Previous studies have linked spontaneous nucleotide release with intracellular physiological activities (Gallagher, 2004; Geyti et al., 2008; Kowal et al., 2015; Ostrom et al., 2000), and the ATP released has been suggested to potentiate the P2YR stimulation via autocrine functions (De Ita et al., 2016; Praetorius and Leipziger, 2009), which may result in stronger Ca^{2+} signals in the early G_1-stage, as was observed in the present study. Furthermore, a previous study suggested that secretory extracellular ATP regulates the nuclear function by strengthening nuclear Ca^{2+} concentrations (Elsing et al., 2012). However, further studies on the production and release of ATP and the relationship between its autocrine activities and Ca^{2+} signaling in nucleotide-stimulated G_1-phase cells may help further

delineate these hypotheses.

ATP-mediated $[Ca^{2+}]_i$ mobilization of both *HeLa.S-Fucci2* and fMFB cells was mainly IP_3-dependent. When Ca^{2+} mobilization was inhibited, ATP failed to induce $[Ca^{2+}]_i$ changes in either cell types. In the arcyriaflavin-A treated cohort, $P2Y_{2,4,6}$ agonist UTP (20 μM) induced changes in $[Ca^{2+}]_i$ that were partially inhibited in both cells. In addition, an RT-PCR analysis revealed that the expression of some P2Y receptors, especially $P2Y_2$, IP_3R_1 and IP_3R_3, was decreased in *HeLa.S-Fucci2* cells. Our UTP results were consistent with the view that the synchronized cohorts had a reduced expression these receptors, especially $P2Y_2$. In the fMFB cells, an RT-PCR analysis revealed that the expression of $P2Y_6$ receptor and IP_3R_2 was decreased. As shown in Figs. 3 and 7, there was little difference in the wave pattern between the *HeLa.S-Fucci2* and fMFB cells, despite the observed differences in the receptor expression. This result may be because the expression of some receptors isoforms differed by cell types while still producing some similar responses with differences in the strength of Ca^{2+} signal. Based on this reasoning, the pattern of $[Ca^{2+}]_i$ changes in G_1-interphase cells may be slightly similar in each type of cells. However, further experiments will be required to clarify the relationship between the mechanism of $[Ca^{2+}]_i$ changes and IP_3R expression in both cell types.

Calcium signaling requires the expression and integration of receptors for particular responses. In the present study, we found that P2YR and IP_3R expression varied in G_1-interphase cells. This phenomenon may also be responsible for the differences observed in the Ca^{2+} signals. The expression of immediate-early genes in the G_1-stage and the phosphorylation of Rb are regulated by Ca^{2+} (Dolmetsch, 2003; Pande et al., 1996; Parkash and Asotra, 2010; Takuwa et al., 1993), resulting in cell growth control during the early G_1-stage as the cell prepares for cycle progression. Putative regulatory genes were previously shown to have altered expression profiles, suggesting mechanisms for the increased or decreased sensitivity of the signaling pathway as a response to ligands (Almagro et al., 2014). The down-regulated expression of genes encoding enzymes involved in microtubule-driven movements affected cell division and expansion as well as intracellular organization (Almagro et al., 2014; Pontin et al., 2010). This hypothesis may be applicable to the results of the present study, since the cells were completely organized during the late G_1-stage but were still growing in the early G_1-stage and the cytoskeleton was more actively involved in cell growth. Therefore, the relevant regulatory genes up-regulate the expression of receptors during the early G_1-phase and down-regulate it during the late G_1-phase, perhaps therefore accounting for variations in the Ca^{2+} signals as a reflection of the observed alterations in the P2YR and IP_3R expression. In addition, Goree et al. reported variations in the expression of purinergic receptors in cultured human liver cell lines and suggested that this was due to the different origins of these cells (Goree et al., 2014). In our RT-PCR results, there were some changes in the expression of receptors, especially P2Y and IP_3R, on the endoplasmic and nucleoplasmic reticulum. Therefore, there may have been some differences in the $[Ca^{2+}]_i$ dynamics in some phases (early, intermediate, or late) of the G_1-phase in the experiment, as shown in Fig. 3. However, our results showed that this was also due to the cell cycle stage of the individual cells. These results are consistent with the previous findings of Okuda et al., which suggested that the expression of the receptors fluctuates periodically in correlation with cell proliferation (Okuda et al., 2003).

In conclusion, the results of the present study highlight the relationship between gene expression and Ca^{2+} signals for physiological activities, as well as Ca^{2+} signals between the nucleoplasm and cytoplasm in early-to-late G_1-phase cells. Further investigations into the mechanisms underlying the different manners of involvement of regulatory elements of the cell cycle in P2YR- and IP_3R-mediated gene expression and activation are strongly recommended. It is tempting to speculate that the inactivation of the transcription factor E2F in the G_1-stage, by remaining attached to Rb (Rb-E2F complex), is the key factor leading to the failure or down-regulated expression of some receptors, thereby allowing the cell to grow before progressing to subsequent stages.

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Conflict of Interest

The authors declare that there are no conflicts of interest that may be perceived as prejudicing the impartiality of this work.

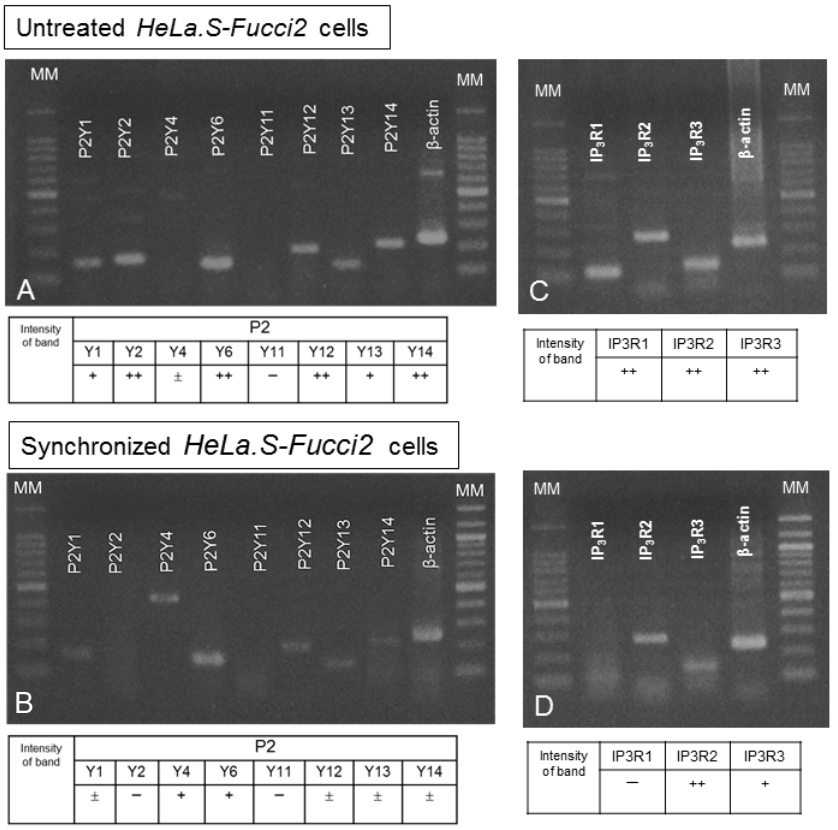

Fig. 5. Expression characteristics of P2YR and IP$_3$R isoform mRNAs in *HeLa.S-Fucci2* cells by RT-PCR. A and C: Untreated cells and B and D: cells treated with 3 μM of arcyriaflavin-A. β-actin was used as a positive control. P2YR expression was graded from (-), where the PCR product was undetectable by ethidium bromide staining of the agarose gel, to (++), where a very strong band was detected. MM: molecular marker. The photos are representative of the results obtained using cells from three independent experiments.

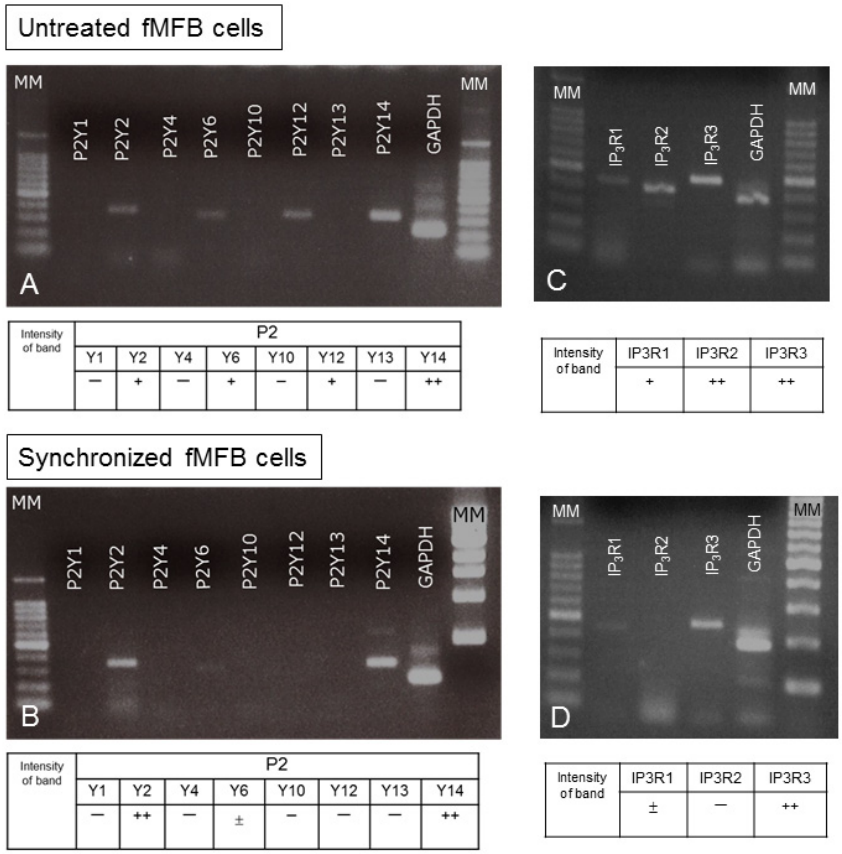

Fig. 6. Expression characteristics of P2YR and IP$_3$R isoform mRNAs in fFMB cells by RT-PCR. A and C: Untreated fMFB cells. B and D: Cells treated with 3 μM of arcyriaflavin-A. GADPH was used as a positive control. P2YR expression was graded from (-), where the PCR product was undetectable by ethidium bromide staining of the agarose gel, to (++), where a very strong band was detected. MM: molecular marker.

Receptor		Gene Sequence 5'- 3'	Position	Accession code	Amplicon (bp)
P2Y receptors					
P2RY1	Sense	AAAACTAGCCCCCTGCAACT	2304	NM002563	153
	Antisense	GATCTGATGCCGGATGAACT	2437		
P2RY2	Sense	CCACCTGCCTTCTCACTAGC	2337	NM176072	163
	Antisense	TGGGAAATCTCAAGGACTGG	2480		
P2RY4	Sense	CGTCTTCTCGCCTCCGCTCTCT	890	NM002565	433
	Antisense	GCCCTGCACTCATCCCCTTTTCT	1300		
P2RY6	Sense	AGCTGGGCATGGAGTTAAGA	1351	NM176797	139
	Antisense	GCTGACTGGGACCTCTCAAG	1470		
P2RY11	Sense	CCTCTACGCCAGCTCCTATG	966	NM002566	211
	Antisense	CACTGCGGCCATGTAGAGTA	1157		
P2RY12	Sense	TTTGCCCGAATTCCTTACAC	1060	NM022788	192
	Antisense	ATTGGGGCACTTCAGCATAC	1232		
P2RY13	Sense	CCCCTGGTACACTTGGAAGA	2496	NM176894	125
	Antisense	TACAGAGGAGGGGGTGATTG	2601		
P2RY14	Sense	TCTTTGGGCTCATCAGCTTT	620	NM014879	213
	Antisense	TCCGTCCCAGTTCACTTTTC	813		
IP_3 receptors					
IP_3R1	Sense	TCTGGGCGCTTTCAATGTAT	7159	D26070	113
	Antisense	AGAGGAACTCAACATCCAGAACC	7249		
IP_3R2	Sense	GACCCAACAGAATACAC	8225	D26350	274
	Antisense	CATGGGGTGTGTTTGATC	8481		
IP_3R3	Sense	AGGTGATCCTGAATCCTGTCAA	780	D26351	142
	Antisense	TCCCGAAACTGCATAAACAGGT	900		
β-actin	Sense	GGACTTCGAGCAAGAGATGG	747	NM001101	234
	Antisense	AGCACTGTGTTGGCGTACAG	961		

Table 1: Primers used for RT-PCR of mRNA from human P2YRs and IP_3Rs. The primers of P2Y were the same as those used by Goree et al. and Kawano et al. The primers of IP_3 were the same as those used by Murthy et al. and Kawano et al. All primers were assessed for specificity using BLAST searches (http://www.ncbi.nlm.nih.gov/blast).

Receptor		Gene Sequence 5'- 3'	Position	Accession code	Amplicon (bp)
P2Y receptors					
P2RY1	Sense	TTTTGTAACATGGTCACAAGACATCCC	1804	NM008772	381
	Antisense	AGTGGCCACGTCACGGTTTT	2184		
P2RY2	Sense	GCTCCGTCATGCTGGGTCT	1071	NM008773	360
	Antisense	CTCGGGCAAAGCGGACAA	1430		
P2RY4	Sense	ACTGGAACTAAGATGGTGCTCCT	141	NM020621	559
	Antisense	GCAGATGCCCATGTAGCGGT	699		
P2RY6	Sense	AGCCCACCCATCCTGTCT	941	NM183168	323
	Antisense	GGCCGAGTGCCTTTGTAG	1263		
P2RY10	Sense	CTTGACATGCATTAGCCTTCAG	650	NM172435	578
	Antisense	GAGCTTCCATGACGATAGTTG	1249		
P2RY12	Sense	CCATTGACCGCTACCTGA	730	NM027571	334
	Antisense	GGAACTTTGGCTGAACCC	1063		
P2RY13	Sense	CTATGAGACGATGTATGTGGGTAT	382	NM028808	379
	Antisense	CTTGTGCCTGCTGTCCTTAC	760		
P2RY14	Sense	CCTTGCTGTCCCAAACAT	976	NM133200	337
	Antisense	ACCTTCCGTCTGACTCTTT	1312		
IP_3 receptors					
IP_3R1	Sense	CACCGCGGCAGAGATTGACAC	2180	NM010585	449
	Antisense	CCAGCTGCCCGGAGATTTC	2610		
IP_3R2	Sense	CTGGGGCCAACGCTAATACTACTT	6910	NM010586	412
	Antisense	GAACCCCGTGATTACCTGTGACTG	7298		
IP_3R3	Sense	GCGGGCCTGTGACACTCTACTTAT	7635	NM080553	485
	Antisense	CGCCGCTCACCAGGGACAT	8101		
GAPDH	Sense	AGCCTCGTCCCGTAGACAAA	36	BC083079	379
	Antisense	GAGATGATGACCCTTTTGGC	414		

Table 2: Primers used for RT-PCR of mRNA from mouse P2YRs and IP_3Rs. The primers of P2Y were the same as those used by Ohtani et al. and Zhang et al., and the primers of IP_3 were the same as those used by Hayato et al. The $P2Y_{10}$ primers were the same as those used by Moriguchi-Mori et al. All primers were assessed for specificity using BLAST searches (http://www.ncbi.nlm.nih.gov/blast).

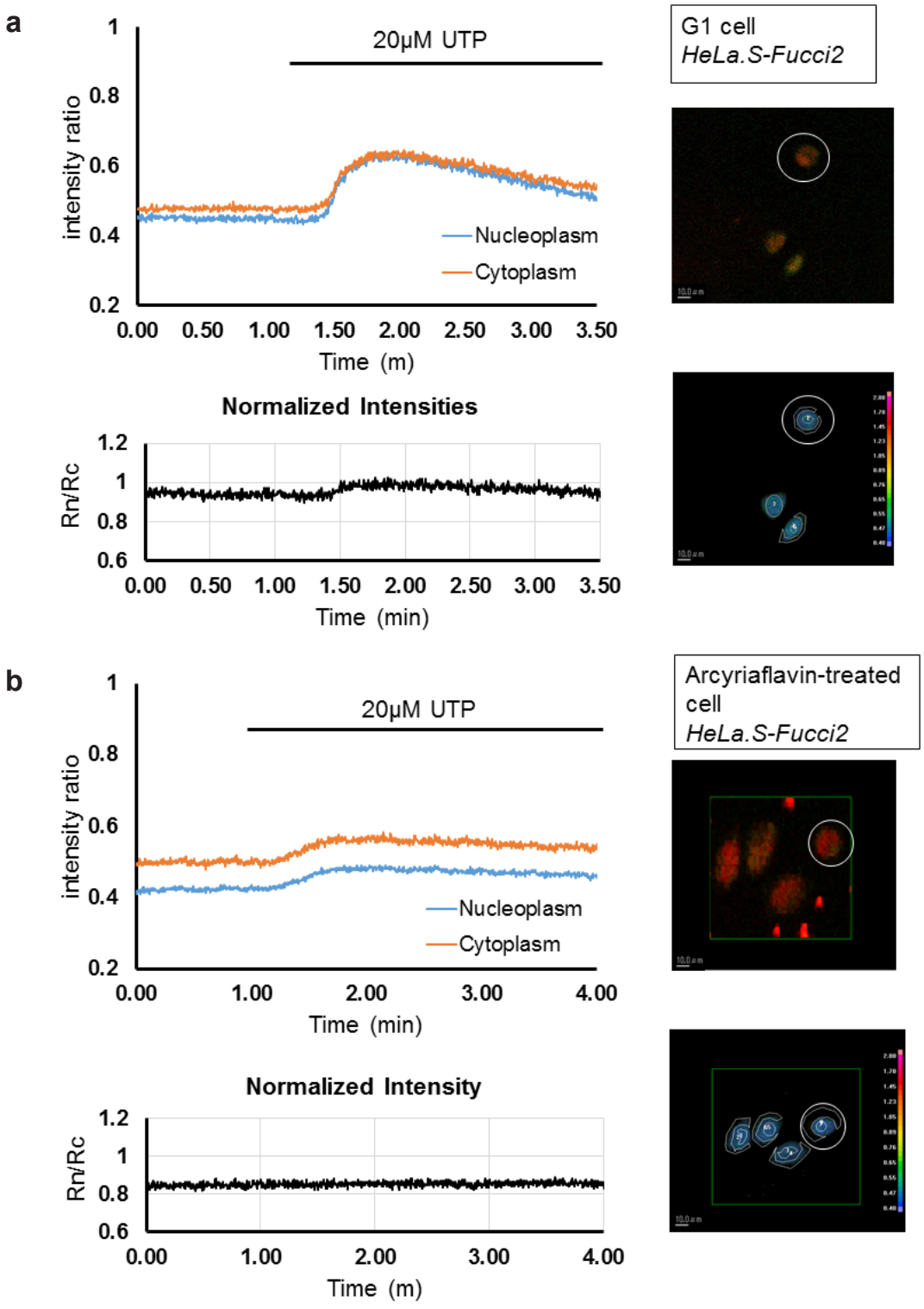

Fig. 7. Time course analyses of UTP-induced $[Ca^{2+}]_i$ dynamics in synchronized and non-synchronized G_1-phase *HeLa.S-Fucci2* cells. A: Non-synchronized cells, B: cells treated with 3 μM arcyriaflavin-A. All cells were challenged by perfusion with 20 μM of extracellular UTP in the absence of extracellular Ca^{2+} and presence of 0.5 M EGTA. The representative records indicate the $[Ca^{2+}]_i$ changes observed between the nucleoplasm and cytoplasm. Rn/Rc: normalized fluorescence ratio at time T, Rn: nucleoplasmic ratio at time T, Rc: cytoplasmic ratio at time T, where R=Ft/Fo. Ft: fluorescence intensity at time T; Fo: fluorescence intensity at time T=0.

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