Multiple Ca^{2+} Signaling Pathways Regulate Intracellular Ca^{2+} Activity in Human Cardiac Fibroblasts

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Abstract

Ca^{2+} signaling pathways are well studied in cardiac myocytes, but not in cardiac fibroblasts. The aim of the present study is to characterize Ca^{2+} signaling pathways in cultured human cardiac fibroblasts using confocal scanning microscope and RT-PCR techniques. It was found that spontaneous intracellular Ca^{2+} ($Ca^{2+}{}_i$) oscillations were present in about 29% of human cardiac fibroblasts, and the number of cells with $Ca^{2+}{}_i$ oscillations was increased to 57.3% by application of 3% fetal bovine serum. $Ca^{2+}{}_i$ oscillations were dependent on Ca^{2+} entry. $Ca^{2+}{}_i$ oscillations were abolished by the store-operated Ca^{2+} (SOC) entry channel blocker La^{3+}, the phospholipase C inhibitor U-73122, and the inositol trisphosphate receptors (IP3Rs) inhibitor 2-aminoethoxydiphenyl borate, but not by ryanodine. The IP3R agonist thimerosal enhanced $Ca^{2+}{}_i$ oscillations. Inhibition of plasma membrane Ca^{2+} pump (PMCA) and Na^+-Ca^{2+} exchanger (NCX) also suppressed $Ca^{2+}{}_i$ oscillations. In addition, the frequency of $Ca^{2+}{}_i$ oscillations was reduced by nifedipine, and increased by Bay K8644 in cells with spontaneous Ca^{2+} oscillations. RT-PCR revealed that mRNAs for IP3R1-3, SERCA1-3, $Ca_V1.2$, NCX3, PMCA1,3,4, TRPC1,3,4,6, STIM1, and Orai1-3, were readily detectable, but not RyRs. Our results demonstrate for the first time that spontaneous $Ca^{2+}{}_i$ oscillations are present in cultured human cardiac fibroblasts and are regulated by multiple Ca^{2+} pathways, which are not identical to those of the well studied contractile cardiomyocytes. This study provides a base for future investigations into how Ca^{2+} signals regulate biological activity in human cardiac fibroblasts and cardiac remodeling under pathological conditions.

Key words. human cardiac fibroblast; Ca^{2+} signaling, intracellular Ca^{2+} oscillations

Introduction

Cardiac fibroblasts account for more than 50% of cells in heart, surrounding myocytes and bridge ‘the voids’ between myocardial tissue layers. Under normal circumstances, cardiac fibroblasts are believed to play an important role: in the maintenance of myocardial structure including extracellular matrix homeostasis and the production of growth factors, cytokines, and matrix metalloproteinases (Baudino et al, 2006). However, during cardiovascular disease, cardiac fibroblasts play a crucial role in myocardial remodeling, including cardiomyocyte hypertrophy, migration and proliferation of fibroblasts, and alterations in deposition and composition of the extracellular matrix. Excessive proliferation and increase in extracellular matrix protein result in fibrosis: subsequent myocardial stiffening that can lead to cardiac dysfunction (Brown et al, 2005;Manabe et al, 2002;Weber and Brilla, 1991). However, the cellular biology and physiology of human cardiac fibroblasts are not fully understood.

The cytosolic free calcium ion (Ca^{2+}_i) functions as a highly versatile second messenger in virtually all types of eukaryotic cells. Ca^{2+}_i regulates a wide range of cell functions including excitation-contraction coupling, excitation-secretion coupling, gene transcription, cell growth, differentiation, apoptosis, membrane fusion, and ion channel activation (Berridge et al, 2000). In most eukaryotic cells, Ca^{2+} is released from the internal store and initiates Ca^{2+} entry across plasma membrane (Berridge et al, 2000). In addition, Ca^{2+} extrusion systems maintain a nanomolar level of Ca^{2+}_i concentration (Herchuelz et al, 2002;Strehler et al, 2007). However, Ca^{2+} signals in cardiac fibroblasts are not as well studied as those as in cardiac myocytes (Bers and Guo, 2005;Grueter et al, 2007;Wang et al, 2004). Little information is available in the literature about the Ca^{2+} signaling pathways in human cardiac fibroblasts; the present study was therefore to characterize Ca^{2+} signaling pathways in cultured human cardiac fibroblasts.

Material and methods

Cell cultures

Human cardiac fibroblasts (adult ventrical, Catalog# 6310) were purchased from ScienCell Research Laboratory (San Diego, CA). The cells were cultured as monolayers in completed DMEM containing 10% fetal bovine serum (Invitrogen, Hong Kong) and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) at 37°C in a humidified atmosphere of 95% air, 5% CO_2. Cells used in this study were from the early passages 2 to 6 to limit the possible variations in Ca^{2+} signals and gene expression (Li et al, 2009).

Ca^{2+}_i measurements

The cells were loaded with 5 μM fluo-3 AM (Biotium Inc., CA) for 30 min at 37°C and incubated in physiological bath solution for 30 min. The bath solution contained (in mM): NaCl 140, NaH_2PO_4, 0.33, KCl 5.0, $MgCl_2$ 1.0, glucose 10, HEPES 10, $CaCl_2$ 2.0. The pH was adjusted to 7.3 with NaOH. Fluo-3 AM was excited at 488 nm and the emission was detected at 506 nm. Ca^{2+}_i concentration ($[Ca^{2+}]_i$) in human cardiac fibroblasts was monitored using a confocal scanning microscope (Olympus FV300; Tokyo, Japan) at room temperature (22-24 °C). $[Ca^{2+}]_i$ was calibrated with a modified procedure as described previously (Merritt et al, 1990). Briefly, ionomycin 2 μM was applied in the end of the experiment to induce a maximal increase of $[Ca^{2+}]_i$, then a Ca^{2+}-free bath solution with 5 mM EGTA was used to reduce the

$[Ca^{2+}]_i$ to the minimum. The free $[Ca^{2+}]_i$ was then calculated by the equation: $[Ca^{2+}]_i = Kd\ [(F-F_{min})/(F_{max}-F)]$, where the Kd is the dissociation constant value of a fluorescence (Kd: ~390 nM for fluo-3), F is the measured fluorescence value, F_{max} is the fluorescence value with ionomycin, and F_{min} is the fluorescence value with Ca^{2+}-free bath solution containing 5 mM EGTA.

Messenger RNA determination

The messenger RNA was examined using the reverse transcription-polymerase chain reaction (RT-PCR) technique as described previously (Li et al, 2006;Li et al, 2005). Briefly, total RNA was extracted from human cardiac fibroblasts using Trizol reagent (Invitrogen), and further treated with DNase I (Invitrogen) for 30 min at 37°C, then heated to 75°C for 5 min and finally cooled to 4°C to remove genomic DNA (Gao et al, 2007). Reverse transcription was performed using a RT system (Promega, Madison, WI) in a 20 µl reaction mixture. A total of 2 µg RNA was used in the reaction, and a random hexamer primer was used for the initiation of cDNA synthesis. After the RT procedure, the reaction mixture (cDNA) was used for PCR.

PCR was performed with thermal cycling conditions of 94°C for 2 min followed by 35 cycles at 94°C for 45 s, 55-58°C for 45 s, and 72°C for 1 min using a Promega PCR kit and oligonucleotide primers as shown in Table 1. This was followed by a final extension at 72°C (10 minutes) to ensure complete product extension. The PCR products were electrophoresed through 1.5% agarose gels and visualized under UV transilluminator (BioRad, Hercules, CA) after staining with ethidium bromide.

Statistical analysis

Categorical data of the present observation were analyzed with Chi-square (χ^2) test. Group data are expressed as means±SEM. Values of $P<0.05$ were considered to be statistically significant.

Results

Intracellular Ca^{2+} activity

The intracellular calcium (Ca^{2+}_i) activity was measured in human cardiac fibroblasts loaded with fluo-3-AM using a confocal microscopy scanning technique. The signals were recorded every 10 s. It was found that 29.4% of cells (221 out of 752 cells) showed spontaneous oscillations of Ca^{2+}_i in a standard bath solution containing 2.0 mM Ca^{2+} without any stimulation, and some cells remained quiescent without change of Ca^{2+}_i (Fig. 1A and 1B). Percentage of cells with Ca^{2+}_i oscillations was increased to 57.3% (55 out of 96 cells, $P<0.05$ vs FBS-free) by employing 3% FBS in the bath solution (Fig. 1C). Resting $[Ca^{2+}]_i$ (30-80 nM), and frequency (0.2-0.4 oscillation/min) and amplitude (100-350 nM) of Ca^{2+}_i oscillations were variable from cell to cell in human cardiac fibroblasts.

Ca^{2+} entry pathways

To determine whether the spontaneous Ca^{2+}_i oscillations are dependent on external Ca^{2+} entry, the cells were exposed to a Ca^{2+}-free solution containing 3.0 mM EDTA. Spontaneous Ca^{2+}_i oscillations disappeared and the resting $[Ca^{2+}]_i$ was reduced to the minimum (close to 0 nM) under Ca^{2+}-free conditions, and recovered when Ca^{2+} was re-applied in the bath medium (Fig. 2A, n=12 cells). These results suggest that Ca^{2+} influx was required for Ca^{2+}_i oscillations.

The voltage-gated L-type Ca^{2+} channels play important roles in excitation-contraction coupling in cardiac myocytes (Bers and Guo, 2005;Grueter et al, 2007). To investigate whether L-type Ca^{2+} channels mediate the Ca^{2+} influx in human cardiac fibroblasts, we tested the effects of L-type Ca^{2+} channel blocker nifedipine and the L-type Ca^{2+} channel activator Bay K8644. Nifedipine (10 μM) slowed the frequency of Ca^{2+}_i oscillations (Fig. 2B) in 70.6% of cells (12 of 17 cells, while Bay K8644 (10 μM) increased (Fig. 2C) in 75% of cells (6 of 8 cells). In a few cells (n = 3), nifedipine slightly increased the duration of Ca^{2+}_i oscillations (data not shown). Mean values of the frequency change of Ca^{2+}_i oscillations are illustrated in Fig. 2D. Nifedipine (10 μM) decreased the Ca^{2+}_i oscillation frequency from 0.303 ± 0.06 oscillation/min of control to 0.23 ± 0.07 oscillation/min ($P<0.01$ vs control). Bay K8644 (10 μM) increased Ca^{2+}_i oscillation frequency from 0.29 ± 0.07 oscillation/min to 0.35±0.08 oscillation/min ($P<0.01$ vs control). However, the percentage of cells with Ca^{2+}_i oscillations was not changed by applying either nifedipine or Bay K8644. These results suggest that L-type Ca^{2+} channels regulate the frequency of Ca^{2+}_i oscillations, but can not initiate Ca^{2+}_i oscillations.

SOC entry (or capacitative Ca^{2+} entry) is a dominant Ca^{2+} entry pathway in non-excitable cells. To determine whether SOC Ca^{2+} entry mediates Ca^{2+} influx, the SOC entry channel blocker La^{3+} (Taylor and Broad, 1998) was tested in human cardiac fibroblasts. Fig. 3A displays that La^{3+} at 100 μM completely inhibited Ca^{2+}_i oscillations (n=20). This result suggests that SOC entry, as in other non-excitable cells, is likely the major mediator of Ca^{2+} influx that regulates Ca^{2+}_i oscillations in human cardiac fibroblasts.

The SOC entry in non-excitable cells refers to a phenomenon in which depletion of intracellular Ca^{2+} stores leads to activation of Ca^{2+}-permeable channels on the plasma membrane (Putney, Jr., 2007). To test whether this is the case for human cardiac fibroblasts, we used thapsigargin, a specific inhibitor of sarcoplasmic-endoplasmic reticulum Ca^{2+} pumps (SERCAs) (Sagara and Inesi, 1991), which induces passive depletion of intracellular Ca^{2+} store and thereby activation of the SOC entry (Takemura et al, 1989). The cells were initially incubated in a nominally Ca^{2+}-free solution for 5 min, and then exposed to the solution containing 1 μM thapsigargin. Thapsigargin induced a rapid increase followed by a slow decline of $[Ca^{2+}_i]$, suggesting an increase of $[Ca^{2+}_i]$ caused by depletion of the Ca^{2+} stores. Re-application of external Ca^{2+} produced another increase of $[Ca^{2+}_i]$, mediated by SOC entry channels activated by depletion of intracellular Ca^{2+} stores (Fig. 3B), which was completely prevented (Fig. 3C) or suppressed (Fig. 3D) by 100 μM La^{3+}. Similar results were obtained in a total of 48 cells with (Fig. 3D) or without spontaneous Ca^{2+}_i oscillations (Fig. 3B and 3C).

Mobilization of intracellular Ca^{2+} stores

It is well recognized that the mobilization of intracellular Ca^{2+} stores in cardiac myocytes is mediated by RyRs (Bers and Guo, 2005;Grueter et al, 2007). To investigate whether it is the case for human cardiac fibroblasts, ryanodine was employed to determine whether it would inhibit spontaneous Ca^{2+}_i oscillations. We found that ryanodine (100 μM) had no effect on the spontaneous Ca^{2+}_i oscillations (Fig. 4A, n=12). In addition, the RyRs activator caffeine at 10 mM did not produce either Ca^{2+}_i transient or oscillations (Fig. 4B, n=12). These results suggest that intracellular Ca^{2+} mobilization is not mediated by RyRs in human cardiac fibroblasts.

The phospholipase C inhibitor U73122 and the IP3Rs inhibitor 2-amino-ethoxydiphenyl borate (2-APB) were then tested in human cardiac fibroblasts. U73122 at 5 μM significantly inhibited Ca^{2+}_i oscillations (Fig. 4C, n=17). Inhibition of IP3Rs by 30 μM 2-APB suppressed Ca^{2+}_i oscillations (Fig. 4D, n=16). On the other hand, the IP3Rs activator thimerosal (Bootman et al, 1992) (3 μM) initiated Ca^{2+}_i oscillations in cells without spontaneous Ca^{2+}_i oscillations (Fig. 4E). The prevalence of Ca^{2+} oscillations was increased to 69.8% (67 of 96 cells, $P<0.01$ vs control) with 3 μM, and to 85.7 % (12 of 14 cells, $P<0.01$ vs control) with 10 μM thimerosal.

To investigate whether the Ca^{2+} uptake contributes to Ca^{2+}_i oscillations, the SERCA inhibitor cyclopiazonic acid (Munaron et al, 2004) was tested in human cardiac fibroblasts. Cyclopiazonic acid (10 μM) abolished spontaneous Ca^{2+}_i oscillations (Fig. 5E), similar results were obtained in a total of 16 cells.

Ca^{2+} extrusion system in human cardiac fibroblasts

Plasma membrane Ca^{2+} ATPase (PMCA) has been identified as a main contributor of intracellular Ca^{2+} extrusion (Kip and Strehler, 2003). To determine the effects of Ca^{2+} extrusion systems on Ca^{2+}_i activity in human cardiac fibroblasts, we tested PMCA blocker carboxyeosin (Sedova and Blatter, 1999) on Ca^{2+}_i oscillations. Carboxyeosin (5 μM) caused a sustained increase of $[Ca^{2+}]_i$ (Fig. 5A). Similar results were obtained in a total of 16 cells.

Na^+-Ca^{2+} exchanger (NCX) of mammalian plasma membrane has emerged as another essential Ca^{2+} efflux mechanism in the maintenance of intracellular Ca^{2+} homeostasis (Berridge et al, 2003;Lytton, 2007). To examine if this system participates in Ca^{2+} homeostasis in human cardiac fibroblasts, the NCX blocker Ni^{2+} was applied to the bath medium. Fig. 5B shows that the spontaneous Ca^{2+}_i oscillations were reversibly inhibited by 2 mM Ni^{2+}. Similar results were obtained in a total of 8 cells. Omission of bath Na^+ (Fig. 5C) inhibited Ca^{2+}_i oscillations and induced a slight increase in $[Ca^{2+}]_i$. These results suggest that Na^+-Ca^{2+} exchanger plays a role in extruding Ca^{2+} from the cytoplasm in human cardiac fibroblasts.

Messenger RNA of Ca^{2+} signaling pathways

Molecular identities of Ca^{2+} signaling pathways were examined in human cardiac fibroblasts with RT-PCR using specific primers (Table 1) for IP3Rs, RyRs, SERCAs, PMCAs, NCXs, or SOC entry channels-related genes, etc. Fig. 6 displays the images of mRNA expression for these genes. The mRNA for IP3R1, IP3R2, IP3R3, SERCA1, SERCA2, SERCA3, NCX-3, PMCA1, PMCA3, PMCA4, $Ca_V1.2$, TRPC1, TRPC3, TRPC4, TRPC6, STIM1, Orai1, Orai2, and Orai3 were significant in human cardiac fibroblasts, whereas no significant mRNAs for RyRs genes were found in these cells (Fig. 6A). Figure 6B displays that no significant bands are observed for the positive genes detected in Fig 6A when PCR reactions were performed with total RNA instead of the RT products.

Discussion

Ca^{2+} signals are well studied in cardiac myocytes (Bers and Guo, 2005;Grueter et al, 2007;Wang et al, 2004). However, Ca^{2+} signaling pathways are not well understood in human cardiac fibroblasts. The present study has demonstrated for the first time that the cultured human cardiac fibroblasts exhibit spontaneous Ca^{2+}_i oscillations (29% cells) in physiological solution. Exposure of these cells to a solution containing 3% FBS induces a significant increase of cell number with sustained Ca^{2+} oscillations (57.8% cells). The $Ca^{2+}{}_i$ activity is mediated by multiple Ca^{2+} signaling pathways, including IP3Rs, SERCAs, NCX, PMCAs and SOCs, but not RyRs.

Cytosolic Ca^{2+} oscillations or fluctuations caused by Ca^{2+} mobilizing stimuli have been reported in many types of non-excitable cells including oocytes (Fewtrell, 1993; Kiselyov et al, 1998), pancreatic acinar cells (LeBeau et al, 1999; Osipchuk et al, 1990), liver cells, airway epithelial cells (Zhang and Sanderson, 2003) and insulin-secreting β cell (Schofl et al, 1996). In the present study, spontaneous $Ca^{2+}{}_i$ oscillations were found to be present in cultured human cardiac fibroblasts. $Ca^{2+}{}_i$ oscillations were dependent on Ca^{2+} entry (Figs. 2 and 3).

L-type Ca^{2+} channel has been well characterized in mammalian cardiac myocytes from different species including humans (Li et al, 1999;Li and Nattel, 1997), and L-type Ca^{2+} channel participates in excitation-contraction coupling in cardiac myocytes (Bers and Guo, 2005;Grueter et al, 2007). In the present study, we found that L-type Ca^{2+} channel regulated $Ca^{2+}{}_i$ oscillations in human cardiac fibroblasts. The L-type Ca^{2+} channel blocker nifedipine reduced the frequency of $Ca^{2+}{}_i$ oscillations, but could not stop $Ca^{2+}{}_i$ oscillations in cardiac fibroblasts. On the other hand, the L-type Ca^{2+} channel activator Bay K8644 increased the frequency of $Ca^{2+}{}_i$ oscillations, but could not initiate $Ca^{2+}{}_i$ oscillations (Fig. 2), which suggests that L-type Ca^{2+} channel in human cardiac fibroblasts as in human preadipocytes (Hu et al, 2009), but not like in cardiomyocytes (Bers and Guo, 2005;Grueter et al, 2007), plays a less effect on $Ca^{2+}{}_i$ activity.

Spontaneous $Ca^{2+}{}_i$ oscillations were abolished by the SOC entry channel blocker La^{3+} in human cardiac fibroblasts (Fig. 3A). Depletion of calcium store by thapsigargin activated SOC entry channels (Fig. 3B), and the calcium store depletion-induced increase of $Ca^{2+}{}_i$ was prevented or suppressed by La^{3+} (Fig. 3C and 3D). These results suggest that in human cardiac fibroblasts $Ca^{2+}{}_i$ activity is likely mediated by SOC entry channels.

It is well known that the sarcoplasmic/endoplasmic reticulum, a specialized calcium storing organelle, is intimately involved in regulating Ca^{2+} movements within cells. IP3Rs and RyRs participate in the release of Ca^{2+} from the sarcoplasmic/endoplasmic reticulum (Berridge et al, 2000; Bootman and Berridge, 1995). It is interesting to note that RyRs may not be involved in $Ca^{2+}{}_i$ activity in human cardiac fibroblasts. First, ryanodine (100 μM) had no effect on $Ca^{2+}{}_i$ oscillations. Second, caffeine (10 mM) did not induce Ca^{2+} release from calcium stores (Fig. 4). Third, no mRNA expression of RyRs was detected in human cardiac fibroblasts (Fig. 6A). These are clearly different from those of cardiac myocytes (Bers and Guo, 2005;Grueter et al, 2007), supporting the notion that RyRs contribute to Ca^{2+} release in excitable cells, but not in non-excitable cells (Chakrabarti and Chakrabarti, 2006).

The spontaneous $Ca^{2+}{}_i$ oscillations were inhibited by the PLC inhibitor U73122 (Fig. 4C) or the IP3Rs inhibitor 2-amino-ethoxydiphenyl borate (Fig. 4D). In addition, the IP3Rs activator thimerosal (Bootman et al, 1992) significantly increased the number of cells with $Ca^{2+}{}_i$ oscillations (Fig. 4E). These properties are similar to those observed in mesenchymal stem cells from human or rat bone marrow (Foreman et al, 2006;Kawano et al, 2002) and in human preadipocytes (Hu et al, 2009). In these types of cells, the spontaneous Ca^{2+} oscillations and FBS-induced $Ca^{2+}{}_i$ oscillations were mediated by IP3Rs. Ca^{2+} is a potent regulator of various transcription factors (Crabtree, 2001), and $Ca^{2+}{}_i$ oscillations can increase both the efficacy and specificity of Ca^{2+} regulation (Dolmetsch et al, 1998;Lewis, 2003). Mathematical models have demonstrated potential dependence of Ca^{2+}_i oscillations on both mobilization of stored Ca^{2+} from the endoplasmic reticulum and Ca^{2+} buffering and re-release by mitochondria (Marhl et al, 2000;Grubelnik et al, 2001).

In addition, Ca^{2+} extrusion systems significantly modified the pattern of Ca^{2+}_i activity in human cardiac fibroblasts. Blockade of plasma membrane Ca^{2+} pumps by carboxyeosin caused a sustained increase of Ca^{2+}_i (Fig. 5A). Inhibition of Na^+-Ca^{2+} exchanger with Ni^{2+} reversibly suppressed spontaneous Ca^{2+}_i oscillations (Fig. 5B), while a complete block of Na^+-Ca^{2+} exchanger with removal of extracellular Na^+ decreased spontaneous Ca^{2+}_i oscillations, and caused a slight increase of basal Ca^{2+}_i (Fig. 5D), similar to that observed in human preadipocytes and mesenchymal stem cells (Hu et al, 2009;Kawano et al, 2002). These results suggest that spontaneous Ca^{2+}_i oscillations are dependent on integrated function of these Ca^{2+} homeostasis systems.

In addition, the present study revealed the molecular identities of Ca^{2+} signaling pathways present in human cardiac fibroblasts. We found that mRNAs for IP3R1, IP3R2, IP3R3, SERCA1, SERCA2, NCX3, PMCA1, PMCA3, PMCA4, and Cav1.2 (for L-type Ca^{2+} channel), and TRPC1, TRPC4, TRPC6, STIM1, Orai1, Orai2, Orai3 (for SOC entry channels). However, no significant mRNAs for RyRs genes were found in human cardiac fibroblasts. The molecular identities correlate closely with functional activities of these Ca^{2+} signaling pathways.

The molecular identities of SOC entry channels have not been confirmed. Members of TRPC superfamily have been suspected as the major candidates for SOCs, because of similarities in cation permeability and activation mechanisms (Venkatachalam et al, 2002). Recent experiments demonstrated that stromal interacting molecule 1 (STIM1) is likely the "sensor" of Ca^{2+} within endoplasmic reticulum Ca^{2+} stores, translocating in response to store-depletion into localized areas of endoplasmic reticulum, or "puncta" close to the plasma membrane (Lewis, 2007;Liou et al, 2005). The STIM1 and Orai1 (calcium release-activated calcium modulator 1) proteins function together to mediate the store-operated Ca^{2+} signaling pathway to recognize and transduce the store-dependent signal and mediate entry of Ca^{2+} across the plasma membrane (Hewavitharana et al, 2007;Prakriya et al, 2006;Gwack et al, 2007). Our RT-PCR results suggest that TRPC1, 3, 4, 6, STIM 1 and Orai1, 2 and 3 (Fig. 6A) are likely responsible for the molecular identities of SOC Ca^{2+} entry channels in human cardiac fibroblasts.

Collectively, the present study demonstrated that in human cardiac fibroblasts Ca^{2+} activity is mediated by multiple Ca^{2+} signaling pathways, including IP3Rs, SERCAs, NCX, PMCAs and SOCs, but not RyRs, which is not identical to those of well-studied contractile cardiomyocytes. The results provide a basis for future investigations into how Ca^{2+} signaling regulates biological and physiological activity of human cardiac fibroblasts and cardiac remodeling under pathological conditions.

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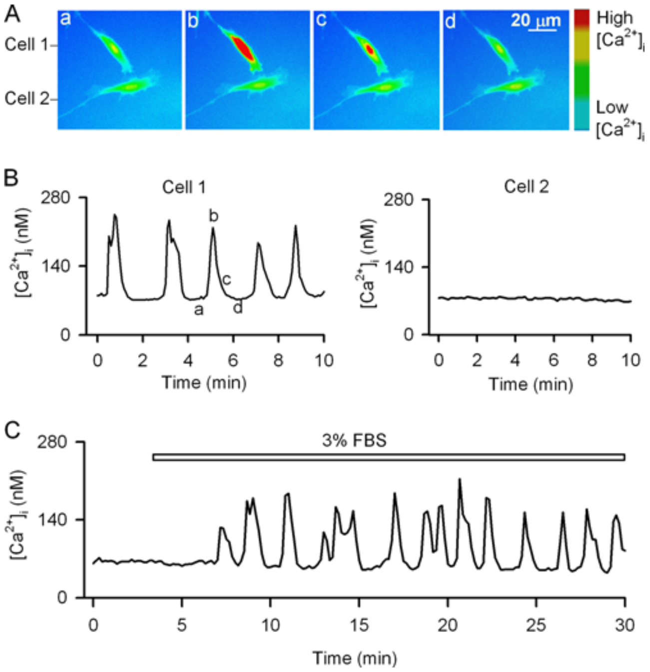

Fig. 1. Characteristics of Ca^{2+}_i oscillations in human cardiac fibroblasts. ***A***. 'Pseudo'-color images show changes in fluorescence intensity (i.e. Ca^{2+}_i) in cell No. 1 and cell No. 2 at time points a, b, c, d, as indicated in panel B. The blue color represents the minimal $[Ca^{2+}]_i$ (close to 0 nM), and the red color represents the highest $[Ca^{2+}]_i$ (>100 nM). ***B.*** Spontaneous Ca^{2+}_i oscillations in cell No. 1 (left panel), but not in cell No. 2 (right panel) during a 10 min recording. ***C.*** Ca^{2+}_i oscillations were initiated by 3% FBS in a human cardiac fibroblast without spontaneous Ca^{2+}_i oscillations.

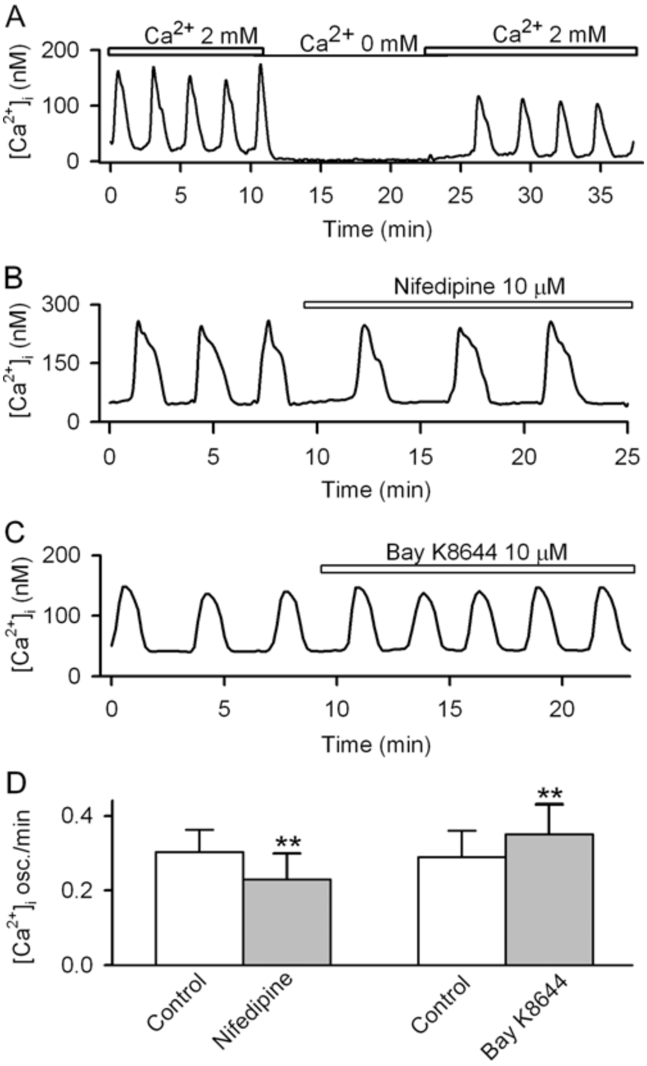

Fig. 2. $Ca^{2+}{}_i$ oscillations and extracellular Ca^{2+}. ***A.*** Spontaneous $Ca^{2+}{}_i$ oscillations disappeared when bath medium Ca^{2+} was removed, and recovered when Ca^{2+} was re-applied (n=22). ***B.*** Nifedipine (10 µM) slowed the frequency of spontaneous $Ca^{2+}{}_i$ oscillations in a representative cell (n=12). ***C.*** Bay K8644 (10 µM) increased the frequency of spontaneous $Ca^{2+}{}_i$ oscillations in another cell (n=6). ***D.*** Summarized changes of $Ca^{2+}{}_i$ oscillation frequency by nifedipine and Bay K8644 (osc., oscillation; **P<0.01 vs control).

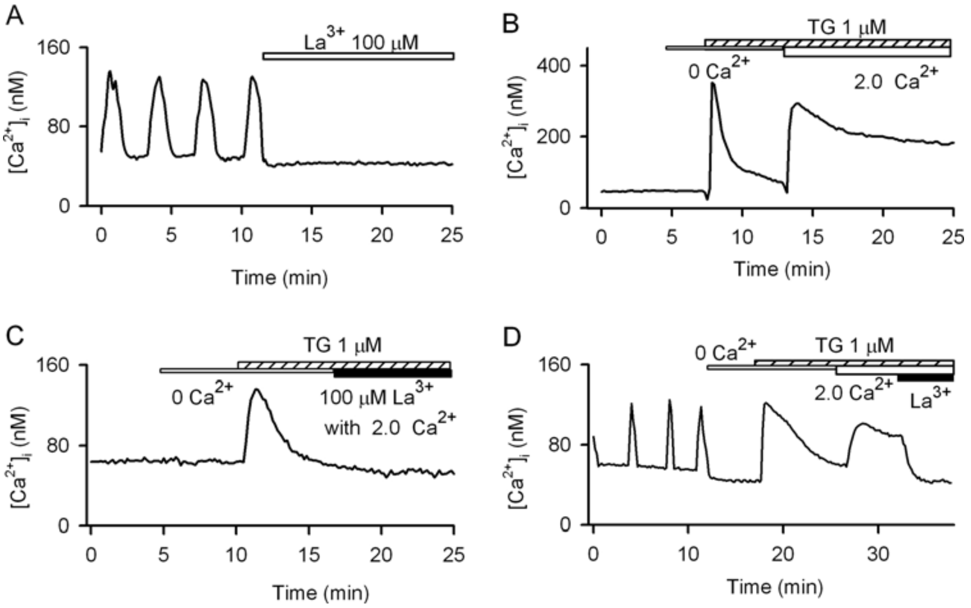

Fig. 3. $Ca^{2+}{}_{i}$ oscillations and store-operated Ca^{2+} (SOC) entry. ***A.*** $Ca^{2+}{}_{i}$ oscillations were fully suppressed by the SOC entry channel blocker La^{3+} (100 μM) in a representative cell (n=20). ***B.*** SOC entry was activated in a representative cell by depletion of Ca^{2+} stores with thapsigargin (TG, 1 μM) and re-application of 2.0 mM Ca^{2+} in the bath solution in a human cardiac fibroblast without Ca^{2+} oscillations. ***C.*** La^{3+} (100 μM) prevented the Ca^{2+} increase caused by reapplied external Ca^{2+}. ***D.*** La^{3+} (100 μM) suppressed the Ca^{2+} increase caused by reapplied external Ca^{2+}.

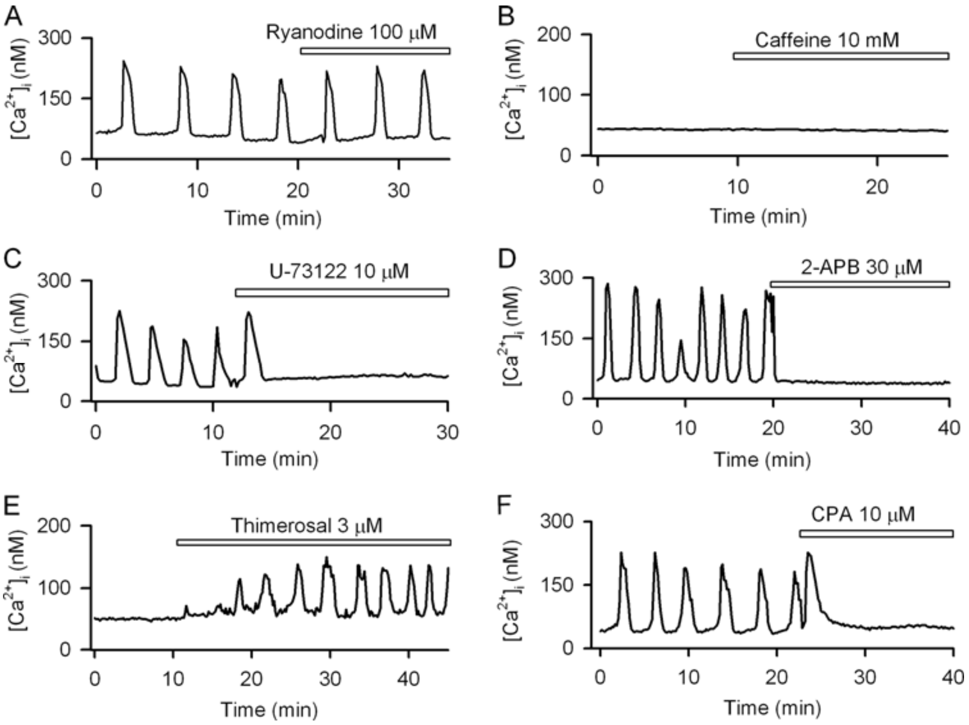

Fig 4: Ca^{2+}_i oscillations and Ca^{2+} release from calcium store. ***A.*** Ryanodine (100 μM) had no effect on spontaneous Ca^{2+}_i oscillations in a representative cell. ***B.*** The ryanodine receptor activator caffeine (10 mM) did not increase Ca^{2+}_i in human cardiac fibroblasts. ***C.*** The PLC-β inhibitor U-73122 (10 μM) suppressed spontaneous Ca^{2+}_i oscillations in a typical experiment. ***D.*** The IP3Rs inhibitor 2-aminoethoxydiphenyl borate (2-APB, 30 μM) inhibited Ca^{2+}_i oscillations. ***E.*** The IP3Rs activator thimerosal (3 μM) initiated Ca^{2+}_i oscillations in cells without spontaneous Ca^{2+}_i oscillations. ***F.*** The SERCA inhibitor cyclopiazonic acid (CPA, 10 μM) induced a transient increase of Ca^{2+}_i and suppressed Ca^{2+}_i oscillations.

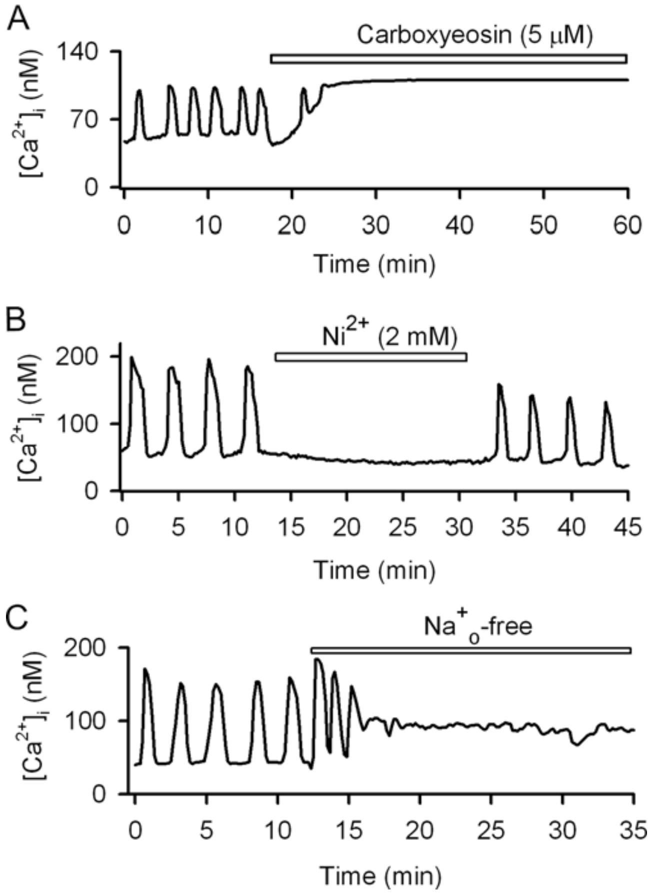

Fig 5: Ca^{2+} extrusion system and $Ca^{2+}{}_i$ oscillations. ***A.*** The plasma membrane Ca^{2+} pump (PMCA) blocker carboxyeosin (5 µM) caused a sustained elevation of $[Ca^{2+}{}_i]$ in a typical experiment. ***B.*** The Na^+-Ca^{2+} exchanger inhibitor Ni^{2+} (2 mM) reversibly inhibited $Ca^{2+}{}_i$ oscillations. ***C.*** Suppression of Na^+-Ca^{2+} exchanger by the removal of bath medium Na^+ decreased $Ca^{2+}{}_i$ oscillations and caused a slight increase in $[Ca^{2+}{}_i]$.

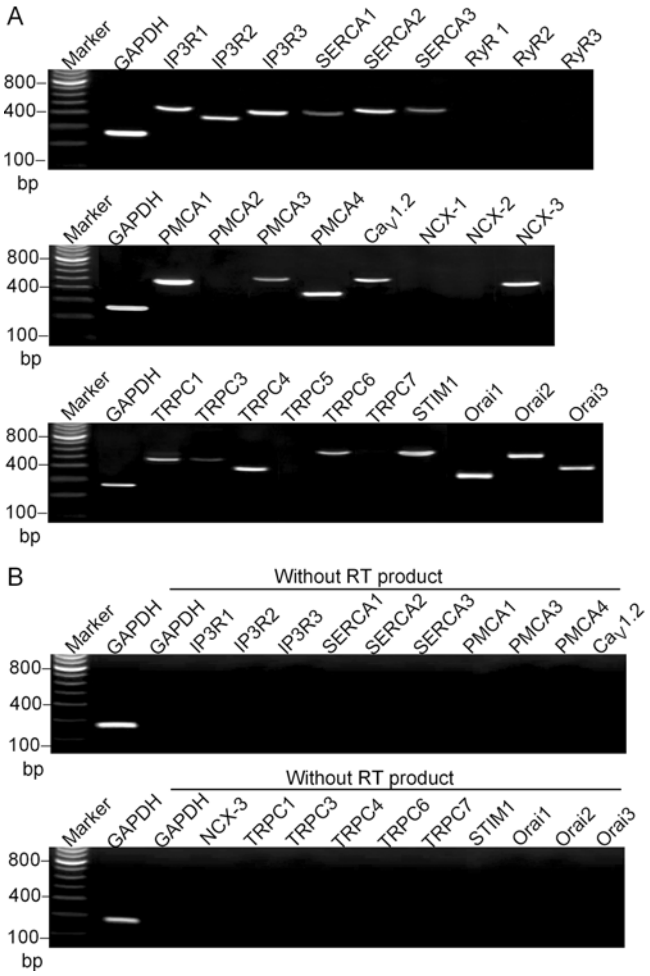

Fig 6. Gene expression of Ca^{2+} signaling pathways. **A.** Images of cDNA bands of PCR products: IP3R1, IP3R2, IP3R3, SERCA1, SERCA2, SERCA2, NCX3, PMCA1, PMCA3, PMCA4, Cav1.2, TRPC1, TRPC4, TRPC6, STIM1 and Orai1, Orai2, Orai3, but not RyRs, are significant in human cardiac fibroblasts. **B.** No significant bands were observed for the positive genes detected in A when total RNA was used for PCR.