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Nuclear Ca^{2+} sparks and waves mediated by inositol 1,4,5-trisphosphate receptors in neonatal rat cardiomyocytes

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Abstract

Dynamic nuclear Ca^{2+} signals play pivotal roles in diverse cellular functions including gene transcription, cell growth, differentiation, and apoptosis. Here we report a novel nuclear Ca^{2+} regulatory mechanism mediated by inositol 1,4,5-trisphosphate receptors (IP_3Rs) around the nucleus in developing cardiac myocytes. Activation of IP_3Rs by α_1 -adrenergic receptor ($\alpha_1\text{AR}$) stimulation or by IP_3 application (in saponin-permeabilized cells) increases Ca^{2+} spark frequency preferentially in the region around the nucleus in neonatal rat ventricular myocytes. A nuclear enrichment of IP_3R distribution supports the higher responsiveness of Ca^{2+} release in this particular region. Strikingly, we observed “nuclear Ca^{2+} waves” that engulf the entire nucleus without spreading into the bulk cytosol. $\alpha_1\text{AR}$ stimulation enhances the occurrence of nuclear Ca^{2+} waves and confers them the ability to trigger cytosolic Ca^{2+} waves via IP_3R -dependent pathways. This finding accounts, at least partly, for a profound frequency-dependent modulation of global Ca^{2+} oscillations during $\alpha_1\text{AR}$ stimulation. Thus, IP_3R -mediated Ca^{2+} waves traveling in the nuclear region provide active, autonomous regulation of nuclear Ca^{2+} signaling, which provides for not only the local signal transduction, but also a pacemaker to drive global Ca^{2+} transient in the context of $\alpha_1\text{AR}$ stimulation in developing cardiac myocytes.

Keywords

Cardiomyocytes; Nuclear Ca^{2+} signaling; Inositol 1,4,5-trisphosphate receptors; Ca^{2+} sparks; α_1 adrenergic stimulation

1. Introduction

The nucleoplasm enclosed by the nuclear membrane represents a relatively segregated cellular compartment that harbors fundamental functions such as DNA and RNA metabolism, gene transcription, and nuclear receptor-mediated signal transduction. By regulating these biological

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processes, nuclear Ca^{2+} signaling plays an important role in cell growth, cell differentiation, cell survival and cell death [1–3]. Rise in nuclear Ca^{2+} can be resulted from cytosolic Ca^{2+} transients via passive Ca^{2+} diffusion through the nuclear pore complexes identified in many cell types including cardiomyocytes [4–7]. However, intensive studies have focused on understanding whether and how nuclear Ca^{2+} is regulated independently of cytosolic Ca^{2+} signaling mechanism, which is more important for local nuclear functions [1,2,8]. A direct release of Ca^{2+} from nuclear envelope into the nucleoplasm has been discovered in rat liver nuclei [9,10], suggesting active and autonomous nuclear Ca^{2+} regulation. Recently, observations in some electrically non-excitable cells, such as SKHep1 cells, suggest that protrusions of the nuclear envelope, which bear inositol 1,4,5-trisphosphate receptors (IP_3Rs), extend into nuclear interior, and result in faster and more sensitive Ca^{2+} response upon IP_3R stimulation in the region of nucleoplasm than in cytoplasm [2,11,12]. However, this independent activation of nuclear Ca^{2+} signal has been controversial due to large quantity of coinstantaneous cytosolic Ca^{2+} signaling [13–15]. More recently, in permeabilized rabbit cardiomyocytes nuclear envelope Ca^{2+} has been found to be decreased by IP_3R activator and directly linked with the activation of Ca^{2+} -calmodulin-dependent protein kinase II pathway, a crucial initiator in cardiac hypertrophy [8]. Therefore, independent IP_3 -dependent nuclear Ca^{2+} regulation may exist in both electrically excitable and non-excitable cells. Yet, the exact mechanism that controls the nuclear signaling is not completely understood. Neither is the dynamic regulation of nuclear Ca^{2+} by IP_3Rs observed in intact cells.

Ryanodine receptors (RyRs) mediated Ca^{2+} release in sarcoplasmic reticulum (SR) almost exclusively control the rhythmic contractions in adult mammalian cardiomyocytes via activation of L-type channel Ca^{2+} influx during cardiac cycle, and this Ca^{2+} release process is termed Ca^{2+} -induced Ca^{2+} release (CICR) [16]. By contrast, IP_3Rs , consist of three subtypes referred to as type 1, 2, 3 [17], are commonly used to release intracellular Ca^{2+} in non-excitable cells through IP_3 induced Ca^{2+} release mechanism [18]. In the heart, IP_3Rs are expressed at much lower level than RyRs and their functional roles remain highly controversial. Recently IP_3Rs have been found to be abundantly expressed in embryonic heart and in adult heart under situations of heart failure and chronic arrhythmias [19–21]. Furthermore, in contrast with RyRs , subcellular distribution of IP_3Rs is often detected in the nuclei [8,22,23]. These observations implicate roles of IP_3R -mediated Ca^{2+} signaling in cardiac development or remodeling in response to stress, consistent with the recent finding of IP_3R -mediated nuclear Ca^{2+} release involved in the activation of nuclear signal transduction for cell growth [8].

In this study, we employed neonatal rat ventricular myocytes (NRVMs) to investigate whether autonomous localized nuclear Ca^{2+} signaling exists and how it is regulated by IP_3 . Using the primary cardiomyocytes has several advantages: firstly, spontaneous hierarchical events ranging from local Ca^{2+} waves to global Ca^{2+} transients coexist in single NRVM, affording rich means for intracellular signal study; secondly, IP_3Rs has been found to distribute abundantly in the nucleus [22,23], implying potential local IP_3R -mediated Ca^{2+} signaling; thirdly, NRVMs are in the stage of growth and IP_3 generators such as α_1 adrenergic receptor ($\alpha_1\text{AR}$) agonist are well documented in induction of hypertrophy. By measuring subcellular Ca^{2+} signaling with fluo 4/AM, we found an active and independent IP_3 -dependent nuclear Ca^{2+} mechanism in the developing heart, which not only regulates the local nuclear Ca^{2+} but also acts as a pacemaker for global cytosolic Ca^{2+} signaling sometimes during $\alpha_1\text{AR}$ stimulation.

2. Materials and methods

2.1. Isolation and culture of cardiomyocytes

NRVMs were isolated from 1 to 2-day-old Sprague–Dawley rats by enzymatic digestion with 0.1% trypsin and 0.03% collagenase, as described [24]. After isolation, the cells were plated

onto laminin-coated 35-mm dishes at a density of 0.5×10^3 cells/mm² and cultured for 48 h in the medium of Dulbecco's modified Eagle's medium and Medium 199 (4:1) containing 10% fetal bovine serum, 4 mM L-glutamine, 100 units/ml penicillin/streptomycin, and 0.1 mM 5-bromo-2-deoxyuridine to inhibit fibroblast proliferation. The medium was changed to serum-free 6 h before use to eliminate any stimulation for cell growth in the serum. The cells exhibited >95% positive staining for α -actinin, and were used between 48 and 55 h into culture.

2.2. Reverse transcription-PCR (RT-PCR)

Myocytes were lysed with TRIzol reagent (Invitrogen) and clarified by centrifugation. RT-PCR was performed as previously described [25] with the use of the primers specific for IP₃R type 1 (PI1 ATGACGAAAGGCGAAGAGAA and PI2: AGCAGCCATAGGAGTCATGG), IP₃R type 2 (PII1: GACACCTCAGATCGACAGCA and PII2: TGAGCCCCCATATTTTTCAG), or IP₃R type 3 (PIII1: GACTGCATTACAGGCACCAG and PIII2: TACAGACTGCTTGCGGCTTA), gene expression. RT-PCR reactions were performed with 1 μ g of total RNA and followed by 30 cycles of PCR amplification.

2.3. Immunoblotting

NRVMs were lysed in RIPA buffer containing 1 mM polymethylsulfonyl fluoride, and 2 μ g/ml protease inhibitor cocktail (Santa Cruz) for 1 h on ice. Homogenates were centrifuged for 15 min at $14,000 \times g$ at 4 °C. The supernatant was used at once or stored at -70 °C until used. Lysates of 60 μ g NRVMs were heated for 5 min, resolved on a 3–8% SDS-PAGE gel (Invitrogen) and transferred to nitrocellulose. Membranes were blocked with 5% nonfat milk powder in Tris-buffered saline containing 0.1% (v/v) Tween 20 for 60 min at room temperature. Two groups of specific antibodies for anti type 1, type 2 and type 3 IP₃R (ABR Inc., and Santa Cruz), were used overnight at 4 °C at dilution of 1:500 and 1:100, respectively. The immunoblotted membrane was then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h and immunoreactive bands were detected by using enhanced chemiluminescence.

2.4. Immunocytochemistry

Cardiac myocytes were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 10 min, permeabilized with 0.1% Triton X-100, washed three times in PBS, and blocked in PBS containing 5% bovine serum albumin, 5% horse serum and 0.05% Triton X-100 for 1 h. Specific subtype anti-IP₃R antibodies were used over night at 4 °C at dilution of 1:200 or 1:100 (ABR and Santa Cruz), respectively. Secondary antibodies were anti-rabbit Texas Red (Vector Lab., 1:250) or mice anti-goat CyTM 2 (1:800). Confocal microscopy of immunocytochemical recording was performed using Zeiss LSM 510 laser microscopy.

2.5. IP₃ measurement

Cultured NRVMs were pretreated with specific inhibitor for the indicated duration followed by stimulation with phenylephrine (PE), an agonist of α_1 AR, for 5 min. The reaction was stopped with addition of ice-cold 10% trichloroacetic acid and the acid extract was homogenized and centrifuged for 15 min at $2000 \times g$ at 0 °C. The supernatants were extracted three times with water-saturated diethyl ether and neutralized to pH 7.5 with NaHCO₃. The level of IP₃ was then quantified according to the protocol of the IP₃ [3H] assay system (Amersham Pharmacia Biotech).

2.6. Confocal Ca²⁺ imaging

Intracellular Ca²⁺ ([Ca²⁺]_i) were measured in intact and permeabilized NRVMs with fluorescence laser scanning confocal microscopy. Intact NRVMs were loaded with 4 μ M

fluo-4/AM (Molecular Probes) at 37 °C for 30 min, and then were washed with HEPES-buffered salt solution (mM: NaCl 135, KCl 5, MgCl₂ 1, CaCl₂ 1.8, HEPES 10 and glucose 11, with pH 7.4 adjusted by NaOH) for 20 min. Confocal images of fluo-4 fluorescence (excitation at 488 nm and emission detection at >515 nm) were obtained using Zeiss LSM 510 inverted microscopy equipped with a 63× oil immersion objective (NA 1.4). Time-lapsed (*xy*, 201 ms/frame) or linescan (*xt*, 2 ms/line, 0.15 μm/pixel) images were obtained with 1.5 μm axial resolution. Ultrafast time-lapsed two-dimensional (*xy*) confocal imaging used Confocal Zeiss Live system, at the axial resolution set at 1.0 μm. [Ca²⁺]_i increases are presented as background-subtracted normalized fluorescence (F/F_0) where F is the fluorescence intensity and F_0 is baseline fluorescence determined by averaging 50 images with no activity. Ca²⁺ sparks image data analysis used customer-devised routines coded in the Interactive Data Language (IDL, Research System).

In some experiments, cultured NRVMs were first permeabilized [26] by a 30 s treatment with a 0.01% saponin solution containing (mM): 100 potassium aspartate, 20 KCl, 3 MgATP, 0.81 MgCl₂, 0.5 EGTA, 20 HEPES, and 5 phosphocreatine, 5 creatine phosphokinase, 10 glutathion, pH 7.0 supplemented with 8% Dextran (43 kDa). Ca²⁺ spark measurement was then performed with 30 μM fluo-4 pentapotassium salt (Molecular Probes) in the presence of 100 nM free Ca²⁺ concentration adjusted according to the protocol by Patton (www.stanford.edu/cpatton). All experiments were performed at room temperature (22–24 °C).

2.7. Statistics

Data were analyzed and presented as means ± (S.E.) of n measurements. When appropriate, statistical comparisons between groups were carried out with 2-way paired or unpaired Student's t test or χ^2 test. The accepted level of significance was $p < 0.05$.

3. Results

3.1. Expression and localization of IP₃R_s in NRVMs

To determine possible role of IP₃R_s in cardiac Ca²⁺ signaling in NRVMs, we first examined the expression of IP₃R_s and their subcellular localization. Measurement of mRNA using RT-PCR demonstrated the presence of all three types of IP₃R_s in neonatal rat atria, ventricles, and cultured NRVMs (Fig. 1A). Western blotting analysis using type-specific anti-bodies confirmed the expressions of type 2 and type 3 IP₃R expressions at the protein level, while type 1 IP₃R expression was below detection (Fig. 1B). Similar with the Western blot analysis, type 1 IP₃R signal was undetectable, while type 3 was distributed in cytoplasm and intense perinuclear and intranuclear staining was evident for type 2 IP₃R in immunofluorescence study (Fig. 1C). We further identified distribution of type 2 RyR, the predominant receptor to release Ca²⁺ from SR in adult cardiomyocytes, with bodipy[®]TR-X ryanodine (Molecular Probes) and specific antibody (ABR Inc.). In agreement with the literature [22,27], RyR_s are exclusively located in cytoplasm (Fig. 1C). These results indicate that both IP₃R_s and RyR_s are expressed in NRVMs. While type 3 IP₃R is mostly distributed in cytoplasm, type 2 IP₃R is enriched to the nuclear region, consistent with previous reports [8,22,23,27].

3.2. Ca²⁺ oscillation responses to α_1 AR stimulation

Many signaling cascades converge on the regulation of the second messenger IP₃ formation. Among them, stimulation of Gq protein-coupled receptors such as α_1 AR leads to activation of phospholipase C (PLC), which catalyzes the cleavage of polyphosphoinositide into dual signaling molecules, IP₃ and diacylglycerol. Thus we next used PE, an α_1 AR activator, to determine the activation of the Gq-PLC signaling cascade and IP₃-dependent Ca²⁺ signaling in NRVMs. Initially, formation of IP₃, the physiological messenger of IP₃R-gated Ca²⁺ release, was measured. As in many cell types and also in cultured cardiomyocytes [28], PE increased

the production of IP₃ in a dose-dependent manner and this effect was abolished by U71322, a PLC inhibitor (Fig. 2A).

Then, we measured $[Ca^{2+}]_i$ in fluo-4 loaded NRVMs by confocal microscopy. Due to spontaneous action potentials as the trigger, rhythmic and spontaneous Ca^{2+} oscillations ($6.02 \pm 0.58 \text{ min}^{-1}$; 74% of the cells) were observed. Pre-treatment of cells with either nifedipine (1 μM ; 10 min), a blocker of L-type Ca^{2+} channels, or thapsigargin (2 μM ; 1 h), an inhibitor of SR Ca^{2+} pump ATPase, completely abolished the Ca^{2+} transients, indicating that Ca^{2+} oscillations were closely dependent on both Ca^{2+} entry and release (data not shown). PE at concentration of 10 μM increased spontaneous Ca^{2+} transient frequency to two folds, while the amplitude of Ca^{2+} transients or the basal $[Ca^{2+}]_i$ was not altered (Fig. 2B). To identify which Ca^{2+} release channel was involved in the mediation of transient increment in response to PE, two membrane permeable IP₃R inhibitors, 2-aminoethoxydiphenyl borate (2-APB) or xestospongine C (Xe C) [29,30], and a RyR inhibitor, ryanodine, were used, respectively. 2-APB at concentration of 3 μM , presumably not affecting such Ca^{2+} signaling mechanisms as capacitative Ca^{2+} entry or inhibition of SR Ca^{2+} pump [29,31], and Xe C (10 μM) markedly prevented the PE effect, while the basal $[Ca^{2+}]_i$ and the amplitude of Ca^{2+} transient remained unchanged (Fig. 1B and D). In another hand, inhibiting RyRs with 30–100 μM ryanodine induced a significant increase in basal $[Ca^{2+}]_i$ (23% of control transients; 92.4% of the cells) and a robust decrease in transient amplitude to $50.6 \pm 6.3\%$ ($p < 0.01$ versus control), but the increase in Ca^{2+} transients due to PE stimulation was not affected (Fig. 2C and D), indicating RyRs were not involved in the PE-mediated spontaneous Ca^{2+} oscillations. Therefore, these observations provided the first clue that IP₃R-associated stimulation preferentially modulates the PE-enhanced spontaneous Ca^{2+} signaling in NRVMs.

3.3. Subcellular spark responses to $\alpha_1\text{AR}$ stimulation

For the assessment of subcellular responsiveness to $\alpha_1\text{AR}$ stimulation, we used high-resolution linescan measurement across the nuclear center along the longitude of a single NRVM and measured spatial temporal Ca^{2+} sparks [32] in the absence and presence of PE. The nuclear region was readily identified by its stronger indicator staining delimited by the sharp and bright staining of its nuclear envelope and immediate surrounding SR (Fig. 3A, inset), which was further confirmed by phase contrast micrographs taken with the same cells (refer to Fig. 1C). We defined “nuclear sparks” as sparks whose centers fell within the nucleus and its 1 μm flanking regions in linescan images; the rest was referred to as “cytosolic sparks”. Because cultured NRVMs spread flatten (thickness usually $< 5 \mu\text{m}$ except the nuclear region) and the focal plane thickness was 1.5 μm in this study, most cytosolic sparks can also be considered as subsurface events.

In PE-unstimulated myocytes, cytosolic sparks occurred at a frequency of $1.4 \pm 0.13 \text{ s}^{-1}$ (100 μm), and nuclear sparks displayed a 61% higher rate ($2.26 \pm 0.19 \text{ s}^{-1}$ (100 μm), $n = 42$ cells, $p < 0.05$ versus cytosol). PE (10 μM) increased spark production by 163% and 86.3% in the nuclear and cytosol regions, respectively, rendering 1.9-fold higher nuclear than cytosol spark response (Fig. 3A and B). Nevertheless, the amplitude, width and duration of sparks in both regions were not different and unchanged by PE stimulation (Fig. 3C). Inhibiting IP₃R with 2-APB and Xe C (data not shown), prevented PE-induced the enhancement of Ca^{2+} sparks in both subcellular areas (Fig. 3A and B), while blocking RyRs with 30–100 μM ryanodine caused an increase in basal $[Ca^{2+}]_i$ and abolishment of the spontaneous sparks and PE effect.

3.4. Subcellular spark responses to IP₃ in permeabilized cells

The hyper-responsiveness of nuclear sparks to $\alpha_1\text{AR}$ stimulation may reflect spatial heterogeneity of either IP₃R organization or $\alpha_1\text{AR}$ signaling components. To discriminate these possibilities, we investigated subcellular spark responses to direct activation of IP₃Rs by IP₃,

bypassing α_1 AR. In saponin-permeabilized NRVMs, basal IP₃-independent nuclear and cytosolic spark frequencies were similar (at 100 nM cytosolic Ca²⁺) (Fig. 4A and B). Tetracaine (0.5 mM), a RyR inhibitor, blocked nuclear and cytosolic Ca²⁺ spark activity to 24.6% and 29.2% of control, respectively (Fig. 4B). Addition of 20 μ M IP₃ in the presence of tetracaine blockade restored the spark activity to 85.7% and 68% of control in respective areas; these rescued sparks were abolished by further application of the IP₃R inhibitor 2-APB (Fig. 4A and B).

To evaluate possible regional variation in IP₃R responsiveness to IP₃, we measured dose-responses for nuclear and cytosolic spark production. Typical results and cumulative data are shown in Fig. 4C and D, respectively. The maximal space-time frequencies of nuclear and cytosolic sparks at saturating IP₃ concentrations were 8.4 ± 0.66 and 5.6 ± 0.49 s⁻¹ (100 μ m), respectively. After subtracting the respective IP₃-insensitive basal spark activity, these numbers reflect a 2.5-fold greater IP₃-sensitive Ca²⁺ spark capacity in the nuclear than the cytosolic region. In other words, Ca²⁺ spark response to IP₃ application was spatially heterogeneous as is the case for α_1 AR stimulation. No regional difference was evident in the IP₃-dependence of spark production, for spark frequencies in both regions displayed steep rise between 3 and 10 μ M IP₃ and reached their respective plateau at 10 μ M or higher IP₃ concentrations. Thus, higher local IP₃R density, rather than IP₃ sensitivity, is likely the primary determinant responsible for the hyper-responsiveness of nuclear Ca²⁺ sparks.

3.5. Nuclear Ca²⁺ waves: an autonomous mechanism for nuclear Ca²⁺ regulation and a pacemaker for global Ca²⁺ transients

Taking advantage of ultrafast confocal imaging capability provided by Zeiss LSM Live system, we visualized and analyzed a novel phenomenon of cellular Ca²⁺ dynamics—*nuclear Ca²⁺ waves* that traveled around and were confined to the nucleus and its immediate surrounding SR. Fig. 5, top panel, shows a time-resolved sequence of a typical nuclear Ca²⁺ wave ($n = 18$ cells). Time courses of nuclear and cytosolic Ca²⁺ transients are shown beneath the image panel. It is clear that, after initiation in the nuclear region, the wave propagated across the entire nucleus, and then dissipated without triggering CICR in the bulk cytosol. As a result, nuclear Ca²⁺ transient was developed independently of any substantial Ca²⁺ changes in the bulk cytosol. Inhibiting IP₃R activity with 3 μ M 2-APB abolished the Ca²⁺ wave activity ($n = 15$), suggesting that activation of IP₃Rs is obligatory to the genesis of nuclear Ca²⁺ waves.

During α_1 AR stimulation, approximately 2.3-fold more (0.41 ± 0.06 s⁻¹ versus 0.18 ± 0.04 s⁻¹ in control, $p < 0.01$, $n = 32$ cells) and bigger nuclear waves were found. Fig. 5B shows that small waves in control condition developed into more and bigger waves upon PE stimulation. Furthermore, while most of them engulfed the entire nucleus, without spreading into the cytoplasm, a part of these waves did evolve into a trigger for a reversed pattern of Ca²⁺ oscillations, that is, prior to PE stimulation Ca²⁺ oscillations were initiated at the cytosol and propagated inwardly into the nucleoplasm (Fig. 6A, upper row). During α_1 AR stimulation, however, nuclear region sometimes lit up first, followed by an outward propagation of Ca²⁺ transient (Fig. 6A, lower row; and Fig. 6B, red trace in b). Linescan measurement further visualized subtle kinetics whereby nuclear Ca²⁺ waves trigger global Ca²⁺ oscillations. Fig. 6C, left panel, shows that, in the absence of PE stimulation, nuclear Ca²⁺ waves engulfed the entire nucleus, and were then abruptly aborted before spreading into the cytosol. α_1 AR stimulation by PE not only markedly increased the occurrence of nuclear Ca²⁺ waves, but also enabled them to trigger cytosolic Ca²⁺ waves in a stochastic manner (23 out of 121 nuclear waves versus 0 out of 58 nuclear waves in control, $p < 0.01$ by χ^2 test). These observations indicated that, in addition to independent and autonomous nuclear Ca²⁺ regulation, nuclear Ca²⁺ waves serve a pacemaker function, which, at least in part, contribute for the potentiation of Ca²⁺ oscillations to α_1 AR stimulation in NRVMs.

4. Discussion

Nuclear membrane constitutes a relative independent microenvironment to ensure the performance of the nuclear biological functions. As such, Ca^{2+} signaling in this particular region seems to be independently regulated because different from Ca^{2+} signaling in cytoplasm, an increase in Ca^{2+} in the nucleoplasm, has been found to cause translocation of nuclear protein kinase C to nuclear membrane [2], type II histone deacetylases to cytoplasm via activation of Ca^{2+} -calmodulin-dependent protein kinase II [8,33], and the transcription of some genes [3,34]. Nuclear envelope together with its tubules and invaginations protruding into the nucleus can be regarded as nuclear Ca^{2+} stores [2,5]. More importantly, ample evidence supporting the existence of IP_3Rs as well as IP_3 -producing machinery in the inner nuclear membrane [2,5,8,27] suggest a functional releasable Ca^{2+} store in the nucleus in response to the extranuclear or/and extracellular stimuli. There is, however, elusion and controversy about the functional role of these receptors and the independent Ca^{2+} signaling in this particular region.

The present study uncovered a rather complex architecture of intracellular Ca^{2+} signaling in ventricular myocytes from the developing rat heart. The molecular players comprise both RyRs and IP_3Rs , creating a hybrid RyR- IP_3R system that is also featured by spatial heterogeneity. The nuclear region, including the nuclear envelope, bearing concentrated IP_3Rs (Fig. 1) appears to be of particular importance, which has also been detected in other studies [2,8,22,23,27]. Special features of nuclear IP_3R signaling include greater basal spark rate, higher spark and Ca^{2+} transient responses to IP_3 and $\alpha_1\text{AR}$ stimulation (Figs. 2–4). Most distinctively, the nuclear/perinuclear SR supports the nucleus-bound, IP_3R -dependent Ca^{2+} waves that engulf the nucleoplasm without initiating CICR in the rest of cytoplasm under control condition (Fig. 5A). Even when CICR is favored by $\alpha_1\text{AR}$ -stimulated elevation of IP_3 , the increased majority of nuclear waves remain uncoupled from global CICR (Fig. 5B). This finding reveals that Ca^{2+} signaling is differentially regulated in the nucleus and cytosol in NRVMs, with the nuclear/perinuclear SR effecting an active, relatively autonomous regulation of nuclear Ca^{2+} signaling. Furthermore, the segregation of nuclear waves attests a spatial discontinuity of CICR, and provides a teleological explanation for the nuclear membrane enrichment of IP_3Rs in NRVMs.

Under conditions favoring IP_3R -dependent CICR, e.g. during $\alpha_1\text{AR}$ stimulation, nuclear waves become intracellular pacemakers of global Ca^{2+} oscillations, further shaping up nuclear and cellular Ca^{2+} dynamics (Fig. 6). This explains, at least in part, the most prominent effect of $\alpha_1\text{AR}$ stimulation on spontaneous Ca^{2+} transient frequency shown in Fig. 2 and other study [35]. On one hand, the enhancement of Ca^{2+} signals likely represents important physiological responses to GqCR activation. On the other hand, these extrasystole Ca^{2+} transients are thought to be arrhythmogenic, because of they disturb the biorhythm of the electrical activity of the surface membrane [36]. Hence, the present study substantiates the notion that IP_3R activation is arrhythmogenic in the heart [36,37], even the receptors sited in nucleus.

Although more than one subtypes of IP_3Rs are detected in NRVMs in the present study and others [22,38], type 2 IP_3R is mainly observed in the nuclear region in this study (Fig. 2), and so is in adult rat ventricular myocytes [8,27]. It is obvious that both type 3 (cytosolic distribution) and type 2 (nuclear distribution) IP_3Rs are responding to IP_3 , but nuclear IP_3R appears much more sensitive to IP_3 than cytosolic IP_3Rs (Figs. 3–5). A same situation is also observed in HepG 2 and SKHep 1 cell lines [2,39]. This may be due to the different density of IP_3R subtype distributed in the regions or/and different sensitivity to IP_3 among subtypes. Interestingly, it has been found that type 2 IP_3R is much more sensitive to IP_3 than the other two subtypes [17,39,40]. This might explain why type 2 IP_3R is mostly distributed in the

nucleus [2,8,17,23,34,39,41], probably for providing unique Ca^{2+} signals and regulating specific functions in this particular region.

In summary, this study reveals that Ca^{2+} signaling can be distinctly regulated in the nucleus and cytosol in NRVMs, with the nuclear membrane system effecting as an active, relatively autonomous site for nuclear Ca^{2+} signaling. Activation of IP_3R profoundly modulates the Ca^{2+} signaling in nucleus, which may explain the mechanism(s) at least partly for cardiac arrhythmias and hypertrophy induced by excess IP_3R -linked Gq protein receptor stimulations.

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Fig. 1. Subcellular IP₃R localization. (A) RT-PCR analysis of IP₃R mRNA in cultured NRVms, ventricle and atria tissues. (B) Western blot analysis of NRVm lysate (60 µg) using antibodies specific for pan-IP₃R, type 1, type 2 and type 3 IP₃Rs. (C) Immunocytochemical staining of NRVms with specific antibodies for type 1, type 2 and type 3 IP₃Rs, respectively; RyRs were labeled by bodipyTR-X ryanodine (0.5 µM), as indicated. Phase contrast micrograph in the right panel shows the positions of the nucleus for type 2 IP₃R staining. "N" with arrow marks the position of nucleus. Scale bar: 10 µm. Similar results were obtained in at least three independent experiments.

Fig. 2.

α_1 AR mediated spontaneous Ca^{2+} oscillations. (A) PE stimulation of NRVMs for 3 min elicited a concentration-dependent increase in IP_3 accumulation that was sensitive to the PLC inhibitor U71322 (10 μM , 20 min prior to PE). * $p < 0.05$; ** $p < 0.01$ vs. control (con). ## $p < 0.01$ vs. PE (10 μM) group. $n = 3$ or 4 independent experiments. (B) Spontaneous global Ca^{2+} oscillations prior to and 3 min after PE treatment (upper trace), and representative effect of 2-APB on basal and PE-induced global Ca^{2+} oscillations (lower trace) in myocytes loaded with the Ca^{2+} indicator fluo-4. (C) Typical trace for effect of ryanodine (Rya) on PE-mediated potentiation of Ca^{2+} transients. (D) Effects of 2-APB, Xe C and Rya (at 3, 10 and 100 μM , pretreated cells for 10, 30 and 10 min, respectively) on PE (10 μM)-induced spontaneous Ca^{2+} oscillation frequency. Data were presented as percentage of vehicle control (DMSO, 0.1%). ** $p < 0.01$ vs. DMSO; ## $p < 0.01$ vs. PE group. $n = 6$ –8 experiments.

Fig. 3.

$\alpha_1\text{AR}$ mediated nuclear and cytosolic Ca^{2+} sparks. (A) Typical linescan images of Ca^{2+} sparks under control conditions (con), in the presence of PE alone (3 min), or PE after 2-APB pretreatment (3 μM , added 10 min prior to PE). Inset: Confocal scan line across the nucleus and the cytosol. Nonuniform resting staining of fluo-4 allowed for the identification of the nucleus and its intensely stained surrounding area. The nuclear region in the linescan image is marked "N". (B) Statistics of spark frequency (events/s (100 μm)). $n = 36\text{--}42$ cells. ** $p < 0.01$ vs. control (con); ## $p < 0.01$ vs. PE; + $p < 0.05$ and ++ $p < 0.01$ vs. respective nuclear region. (C) Morphometrics of nuclear and cytosolic Ca^{2+} sparks in NRVMs. Amp, W50 and D50 denote amplitude, width at half peak and duration at half peak of Ca^{2+} spark, respectively. $n = 24$ cells.

Fig. 4.

Spark response to IP₃ in permeabilized NRVMs. Myocytes were permeabilized with 0.01% saponin as described in Section 2. (A) Typical results illustrate suppression of spontaneous sparks by tetracaine (Tetra, 0.5 mM, 2 min), restoration of IP₃R-mediated sparks by 20 μM IP₃ in the presence of the ryanodine blocker, and the abolition of IP₃R sparks by further administration of 2-APB (3 μM). "N" marks a nuclear region and the inset shows linescan position. (B) The statistic data for A. $n = 15-18$ cells. ** $p < 0.01$ vs. control (con); ## $p < 0.01$ vs. Tetra treatment. (C) Typical result of IP₃ (10 μM)-induced Ca²⁺ sparks. (D) Dose-dependent effect of IP₃ on nuclear and cytosolic Ca²⁺ sparks. ** $p < 0.01$ vs. prior to IP₃ addition; ++ $p < 0.01$ vs. nuclear region. Data were obtained 2 min after IP₃ application; each dish of cells was subjected to one dose of IP₃ only. At least 11 cells in 9-11 separated experiments were used for each data point.

Fig. 5.

Nuclear Ca^{2+} sparks and Ca^{2+} waves in NRVMs. (A) Time-resolved 2D (xy) images were obtained with Zeiss Live confocal microscope at rate of 40 frames/s. Regenerative Ca^{2+} release was initiated at 5-o'clock position of the nucleus, traveled across the entire nucleus, but failed to trigger any cytosolic Ca^{2+} waves. Inset: contour plots showing the wavefronts at 25 (innermost), 50, 75, and 100 ms after Ca^{2+} release initiation, respectively. Bottom: time courses of spatially averaged Ca^{2+} transients from the outmost contour (nucleus) and blue color-coded areas (cytosol), respectively. (B) Time-resolved 2D (xy , upper) and linescan images (lower) from a different cell from A shows Ca^{2+} release in the nuclear region was enhanced by PE (10 μM) stimulation. The arrow indicates a small Ca^{2+} release in control condition. The traces were obtained from 2D scanning above and labels "N" and "C" denote nuclear and cytosolic spaces, respectively.

Fig. 6. Nuclear Ca²⁺ waves triggered global CICR during α_1 AR stimulation. (A) Time-lapsed xy confocal images of a myocyte before (upper row, con) and after PE stimulation (lower row). Circles 1 and 2 mark the nuclear region and an equal sized cytosolic region, respectively. Arrow in upper row marks a delay in cytosol-to-nucleus propagation of Ca²⁺ transient; arrow in lower row marks nuclear Ca²⁺ event triggering the cytosolic Ca²⁺ transient. (B) Time courses of local Ca²⁺ dynamics in circles 1 and 2 in A. Boxed regions a and b are shown to the right on an expanded time scale. Arrowheads mark nuclear Ca²⁺ waves. (C) Evolvement of nuclear Ca²⁺ waves. Linescan images (top) and line plots of nuclear (N) and cytosolic Ca²⁺ transients (C) (bottom), prior to (Con), 2 and 3 min after PE (10 μ M) stimulation. Fates of nuclear Ca²⁺ waves include (i) engulfing the entire nucleus without triggering cytosolic Ca²⁺ transients (arrows 1, 3); (ii) aborted after traveling a short distance (arrow 4); (iii) triggering a cytosolic Ca²⁺ wave that swept over half of the cell (arrow 2); (iv) triggering cytosolic Ca²⁺ waves that swept over the entire cell (arrow 5).