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Role of Inositol-1,4,5-Trisphosphate Receptor in the Regulation of Calcium Transients in Neonatal Rat Ventricular Myocytes

Zheng Zeng[1,2,†], Heping Zhang[3,†], Na Lin[4], Man Kang[1,2], Yuanyuan Zheng[1,2], Chen Li[1,2], Pingxiang Xu[1,2], Yongquan Wu[3], and Dali Luo[1,2,*]

[1]*Department of Pharmacology, Capital Medical University, Beijing 100069, P.R. China*
[2]*Beijing Key Laboratory of Metabolic Disorders Related Cardiovascular Diseases, Beijing 100069, P.R. China*
[3]*Department of Cardiology, Beijing Friendship Hospital Affiliated to Capital Medical University, Beijing 100050, P.R. China*
[4]*Institute of Molecular Medicine, Peking University, Beijing 100871, P.R. China*

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Abstract. This study determined the regulatory effect of inositol 1,4,5-trisphosphate receptors (IP_3Rs) on the basal Ca^{2+} transients in cardiomyocytes. In cultured neonatal rat ventricular myocytes (NRVMs) at different densities, we used confocal microscopy to assess the effect of IP_3Rs on the endogenous spontaneous Ca^{2+} oscillations through specific activation of IP_3Rs with myo-IP_3 hexakis (butyryloxymethyl) ester (IP_3BM), a membrane permeable IP_3, and interference of IP_3R expression with shRNA. We found that NRVMs at the monolayer state displayed coordinated Ca^{2+} transients with less rate, shorter duration, and higher amplitude compared to single NRVMs. In addition, monolayer NRVMs exhibited 4 or 10 times more increased Ca^{2+} transients in response to phenylephrine, an α-adrenergic receptor agonist, or IP_3BM than single NRVMs did, while the transient pattern remained unaltered, suggesting that the sensitivity of intracellular Ca^{2+} response to IP_3R activation is different between single and monolayer NRVMs. However, interference of IP_3R expression with shRNA reduced the frequency and amplitude of the spontaneous Ca^{2+} fluctuates similarly in both densities of NRVMs, resembling the effects of ryanodine receptor inhibition by ryanodine or tetracaine. Our findings suggest that IP_3Rs are involved, in part, in the regulation of native Ca^{2+} transients, in profiles of their initiation and Ca^{2+} release extent, in developing cardiomyocytes. In addition, caution should be paid in evaluating the behavior of Ca^{2+} signaling in primary cultured cardiomyocytes at different densities.

Keywords: inositol 1,4,5-trisphosphate receptor, Ca^{2+} transient, ventricular myocyte, ryanodine receptor, gap junction

Introduction

Cells possess a Ca^{2+}-signaling toolkit with many components that can be mixed and matched to create a wide range of spatial and temporal Ca^{2+} signals (1, 2). In heart, intracellular Ca^{2+} signaling plays a critical role in the excitation–contraction coupling, and thus the rhythmic myocardium contractions by generation of global Ca^{2+} transients (3 – 5). Several signaling pathways including the adrenergic receptor pathway and drugs such as digoxin can affect the cytosolic Ca^{2+} transients. In the physiological context, an internal Ca^{2+} signal is derived either from the internal stores, from the extracellular space, or both. There are multiple different plasma-membrane channels that control Ca^{2+} influx in response to various stimuli, while the release of Ca^{2+} from sarcoplasmic reticulum (SR) is controlled by Ca^{2+} itself, or by multiple Ca^{2+} effectors such as ryanodine receptors (RyRs) and IP_3Rs (1, 3, 4, 6).

Ca^{2+} transients are believed to be caused primarily by the cardiac action potential that induces brief pulses of Ca^{2+} entry, and consequently activation of RyRs and mobilization of internal stored Ca^{2+} (1, 4). Compared

†Both authors contribute equally to this work.
*Corresponding author. luodl@ccmu.edu.cn
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with RyRs, IP_3Rs are expressed much less in the heart and seem not so necessary for controlling Ca^{2+} transients and excitation–contraction coupling (7 – 10). However, recent studies have demonstrated that the IP_3/IP_3R signaling pathway is involved in triggering cardiac beats in normal heart (10) and arrhythmias in diseased heart (11, 12). Additionally, in previous studies (8, 13), we have found that inhibition of IP_3Rs with 2-aminoethoxydiphenyl borate (2-APB) significantly affects the rate of spontaneous Ca^{2+} oscillations in cultured neonatal rat ventricular myocytes (NRVMs), but it is not clear whether this effect is specific, and the role of IP_3/IP_3R pathway in the regulation of the Ca^{2+} transient formation process in NRVMs.

In the present study, we addressed these issues by evaluating the frequency, amplitude, and duration of the basal Ca^{2+} transients in single and monolayer NRVMs in response to activation or inhibition of IP_3Rs. The purpose for adopting single NRVM is to eliminate the possible effect of gap junctions on the Ca^{2+} signaling in monolayer NRVMs (13 – 15). We found that like RyRs, IP_3Rs also regulate the basal spontaneous Ca^{2+} activity in NRVMs.

Materials and Methods

Materials

Fluo-4/AM was obtained from Molecular Probes (F-14201; Invitrogen, Inc., Carlsbad, CA, USA). Phenylephrine (PE) was purchased from Sigma-Aldrich (P6126; Tampa, FL, USA). IP_3BM was synthesized as described in the previous study (purity > 95%) (16). Two groups of specific antibodies for anti-pan-IP_3R and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Calbiochem (407143; Frankfort, KY, USA) and Santa Cruz Biotechnology, Inc. (sc-51907; Santa Cruz, CA, USA), respectively. Anti-RyR antibody (ab2868) and anti-connexin43 (Cx43) antibody (ab87645) were purchased from Abcam (Cambridge, UK). Secondary antibody donkey-anti-rabbit (A-21206) and chicken-anti-goat (A-21468) were purchased from Invitrogen. Another secondary antibody goat-anti-mouse was purchased from Santa Cruz Biotechnology (sc-16516). All the reagents used, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin (27250-018), collagenase (17101-015), and 5-bromo-2-deoxyuridine (B23151) were purchased from Invitrogen. Dulbecco's modified Eagle's medium (DMEM, 12100-046) and Medium 199 (M199, 31100035) were purchased from Gibco (Life Technologies, Inc., Rockville, MD, USA).

Isolation and culture of neonatal rat ventricular myocytes

NRVMs were isolated from 1 – 2-day-old Sprague-Dawley rats by enzymatic digestion with 0.1% trypsin and 0.03% collagens, as described (8, 13). After removing cardiac fibroblasts, NRVMs were plated onto 60- or 35-mm dishes at a density of 1×10^6 cells/ml for monolayer or diluted 10-fold for single NRVMs study and cultured in DMEM and M199 (4:1) containing 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, and 0.1 mM 5-bromo-2-deoxyuridine to inhibit fibroblast proliferation for 48 h before use.

Confocal Ca^{2+} imaging

As previously described (8, 13), intracellular Ca^{2+} was measured in single and monolayer NRVMs with fluorescence laser scanning confocal microscopy. NRVMs were loaded with 4 μM fluo-4/AM at 37°C for 30 min, and then they were washed with HEPES-buffered salt solution (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$,10 mM HEPES, and 12 mM glucose, 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 Leica SP5 microscopy equipped with a 63 × oil immersion objective (NA 1.4). Intracellular Ca^{2+} 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. All experiments were performed at room temperature (22°C – 24°C).

Preparation of shRNA against IP_3Rs

Recombinant adenovirus for silencing IP_3Rs was prepared with the BLOCK-iT Adenoviral RNAi Expression System (Invitrogen). The sequences of the oligonucleotides for IP_3R RNA interference were as follows: type 1 IP_3R (IP_3R1): forward, CACCGCACTTGAACCAGAT TATAGACGAATCTATAATCTGGTTCAAGTGC; reverse, AAAAGCACTTGAACCAGATTATAGATT CGTCTATAATCTGGTTCAAGTGC. type 2 IP_3R (IP_3R2): forward, CACCGGTACCAGCTAAACCTCTTTGCG AACAAAGAGGTTTAGCTGGTACC; reverse, AAA AGGTACCAGCTAAACCTCTTTGTTCGCAAA GAGGTTTAGCTGGTACC. type 3 IP_3R (IP_3R3): forward, CACCGCACATGAAGAGCAACAAATACG AATATTTGTTGCTCTTCATGTGC; reverse, AAA AGCACATGAAGAGCAACAAATATTCGTATTT GTTGCTCTTCATGTGC. Adenoviral vector containing a scrambled shRNA sequence at the same multiplicity of infection served as the control; forward 5′-CAC CGCCTGCCGTCCAAAGTTGTAACGAATTACAAC TTTGGACGGCAGGC-3′; reverse, 5′-AAAAGCCTG CCGTCCAAAGTTGTAATTCGTTACAACTTT

GGACGGCAGGC-3′.

For knocking down IP_3R expression, NRVMs were cultured for 24 h, transduced with the combination of the adenovirus carrying each IP_3R isotype silencing gene (IP_3Rs-shRNA, virus = 30 m.o.i. for each, altogether virus = 90 m.o.i.) or with scrambled gene (Scram-shRNA, virus = 90 m.o.i.), and were then further cultured for 48 h.

Western blotting

As previous described (8, 13), NRVMs were lysed in RIPA buffer containing 1 mM polymethylsulfonyl fluoride and 2 μg/ml protease inhibitor cocktail (Santa Cruz) for 1 h on ice. Homogenates were centrifuged for 15 min at 14,000 × *g* at 4°C. Lysates of 60 μg NRVMs were heated for 10 min, resolved on an 8% (for IP_3Rs and RyRs) or 10% (for Cx43) SDS-PAGE gel (Invitrogen), and transferred to nitrocellulose. Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 for 60 min at room temperature. Anti-pan-IP_3R antibody (1:500), anti-RyR antibody (1:4000), anti-Cx43 antibody (1:1500), and anti-GAPDH antibody (1:3000) were used overnight at 4°C, respectively. The immunoblotted membrane was then incubated with horseradish peroxides–conjugated secondary antibody for 1 h and immunoreactive bands were detected by using enhanced chemiluminescence.

Immunocytochemistry

The immunocytochemistry of NRVMs were performed as previously described (8, 13). In brief, NRVMs were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 10 min followed by permeabilization with 0.1% Triton X-100 for 10 min at room temperature. Then after blocking in PBS containing 5% bovine serum albumin for 1 h, anti-Cx43 (1:100) and anti-pan-IP_3R (1:100) were used to double-stain the samples overnight at 4°C. The secondary antibodies Alexa Fluor 488-labeled donkey-anti-rabbit (1:500) and Alexa Fluor 594-labeled chicken-anti-goat (1:500) were used for 1 h at room temperature. The nucleus was labeled with Hoechst 33258 at a final concentration of 1 μg/ml, and the subcellular distribution of IP_3Rs and Cx43 were analyzed by confocal microscopy equipped with a 63 × oil immersion objective (NA 1.4).

Statistics

All values are expressed as means ± standard deviation (S.D.), and n represents the number of measurements. When appropriate, statistical comparisons between groups were carried out with the 2-way paired or unpaired Student's *t*-test for 2 groups and one-way ANOVA test for 3 groups. *P*-value of < 0.05 was considered significant.

Results

Differences in spontaneous Ca^{2+} transients between single and monolayer cultured NRVMs

It has been found that gap junctions in connected cells affect the internal Ca^{2+} activity significantly (13 – 15); thus, we first compared the spontaneous basal Ca^{2+} oscillations between single and monolayer cultured NRVMs using confocal microscopy. In non-stimulated myocytes, due to the spontaneous action potentials as triggers, native and rhythmic Ca^{2+} transients were observed in cultured NRVMs at both densities loaded with fluo-4 (Fig. 1A). The respective dynamic parameters in Ca^{2+} fluctuations were assessed by high-speed line scanning measurement across the center of a myocyte (Fig. 1B). Single NRVMs displayed 2 to 11 beats/min (mean value = 6.87 ± 0.33 beats/min, n = 315 cells) non-synchronized Ca^{2+} transients in 61.6% ± 3.2% of examined cells from 32 independent determinations, while monolayer NRVMs exhibited slower but synchronous oscillations in all the tested NRVMs, with a mean value of 4.23 ± 0.22 beats/min obtained from 23 independent experiments (Fig. 1C). In addition, while the parameters of time to peak were not different (data not shown), the peak amplitude and 90% duration (duration 90) of Ca^{2+} transients in monolayer NRVMs were bigger and shorter than those in single NRVM (Fig. 1: D and E). Moreover, activation of endogenous IP_3Rs with phenylephrine (PE, 10 μM for 2 min), a selective agonist of the α_1-adrenergic receptor, significantly increased the Ca^{2+} transient frequency in both densities of NRVMs, but 4 times more transients were observed in monolayer NRVMs than single NRVMs (Fig. 1: F and G), while the parameters of peak amplitude and duration 90 remained unaltered (date not shown). Similarly, the potency and maximal response of Ca^{2+} transient frequency to PE stimulation from 30 to 100 μM were significant higher in monolayer NRVMs (26.8 ± 2.1 vs. 19.8 ± 1.56, $P < 0.01$) than single cells, although the EC50 values were generally similar (4.1 μM and 4.6 μM in single and monolayer NRVMs, respectively, Fig. 1H). These data indicate that sparse and monolayer cultured NRVMs behave differently in both resting status and upon stimulation.

Effect of IP_3R activation on spontaneous Ca^{2+} transients in single and monolayer cultured NRVMs

To identify that the potential effect of PE on internal Ca^{2+} activity is mediated by IP_3Rs rather than other effector activations such as protein kinase C activation, we further used IP_3BM (exogenous membrane permeant IP_3) to activate IP_3Rs directly (16). As shown in Fig. 2, A and E, treatment of NRVMs with IP_3BM at concentrations of 10 and 20 μM for 6 min induced a dose-dependent

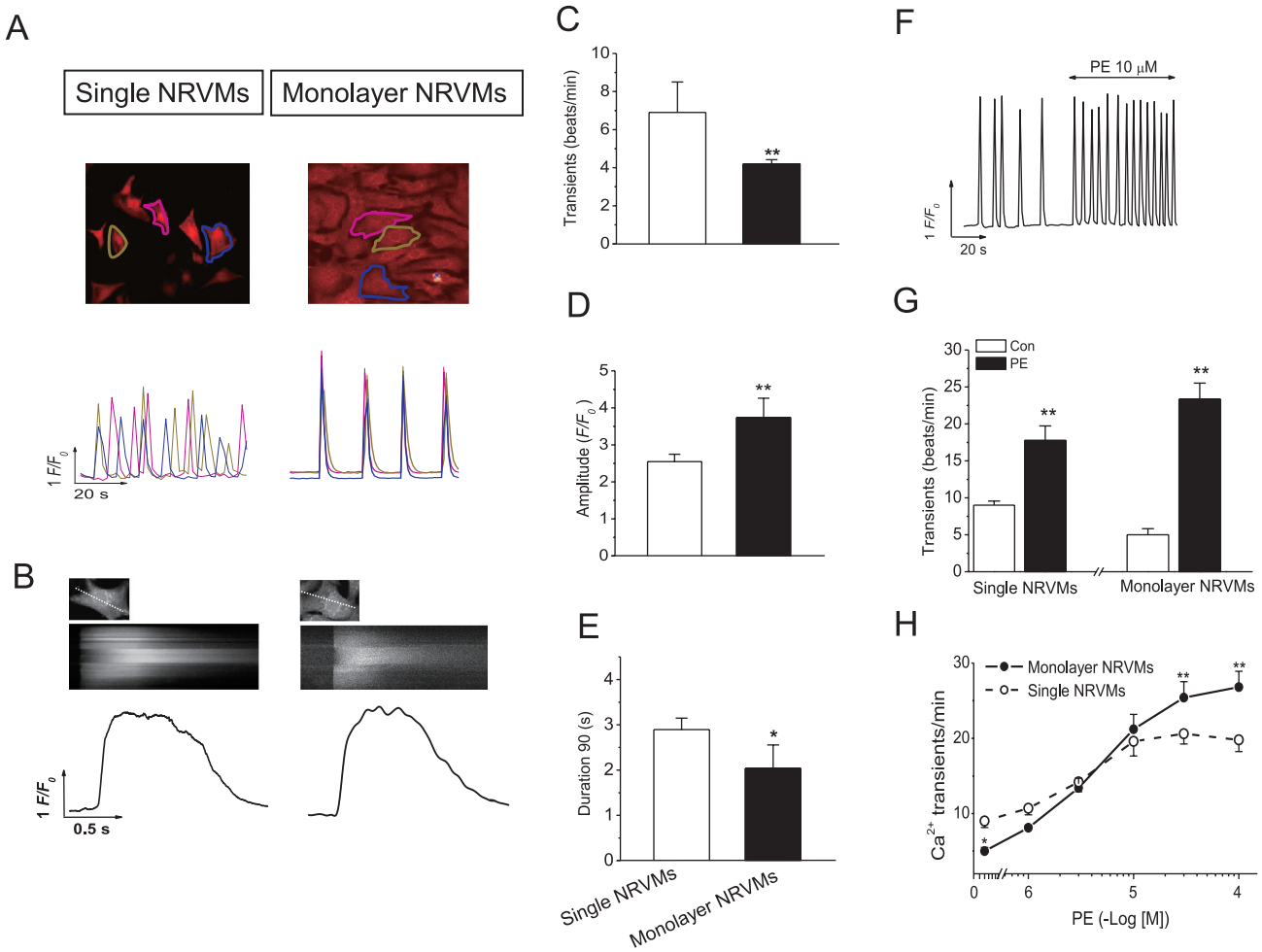

Fig. 1. Differences in spontaneous Ca^{2+} transients between single and monolayer cultured NRVMs. A) The internal Ca^{2+} alterations in NRVMs loaded with the Ca^{2+} indicator fluo4 and the transient traces represented by F/F_0 (lower panel) in single and monolayer NRVMs were monitored by confocal microscopy. B – E) Typical linescan images (scanning across the center of the cell) of Ca^{2+} transient (upper panel) and their traces (lower panel) are shown in B. The statistical data of the Ca^{2+} transient frequency (C) were obtained from 315 and 267 cells in 32 and 23 independent determinations for single and monolayer NRVMs, respectively, while the transient amplitude (D) and duration 90 (E, representing the time for 90% recovery of a Ca^{2+} transient to baseline) as indicated were obtained from 53 and 67 cells in 6 independent determinations for single and monolayer NRVMs, respectively. $*P < 0.05$ and $**P < 0.01$ vs. single NRVMs. F) Typical trace represents the spontaneous Ca^{2+} transients in monolayer NRVMs prior to and after PE (10 μM) treatment for 2 min. G) The statistical data of the Ca^{2+} transients' frequency in single and monolayer NRVMs as indicated were obtained from 32 to 45 cells from 6 independent determinations for each bar in single NRVMs and 10 to 12 independent determinations for each bar in monolayer NRVMs. $**P < 0.05$ vs. control NRVMs (Con). H) Dose-dependent effects of PE on single and monolayer NRVMs' frequency of Ca^{2+} transients were detected from 8 to 12 independent determinations for each point. $*P < 0.05$ and $**P < 0.01$ vs. single NRVMs, respectively.

increase in Ca^{2+} transient rate in both single and monolayer NRVMs, while their amplitude, time to peak, and duration 90 remained unchanged (Fig. 2: B – D and F – H). IP_3BM, like PE treatment, exerted approximately 10 times more potent effect on monolayer NRVMs than on single NRVMs. To exclude the possibility of any change in Ca^{2+}-release channels that may contribute to this different sensitivity between single and monolayer NRVMs, the endogenous expressions of IP_3Rs and RyRs were examined in NRVMs, which showed no difference in their relative abundances between the two density cells after normalization by equal amount loading control protein GAPDH (Fig. 2: I and J).

Thus, the above data showed that the formation of gap junctions may affect the behavior of Ca^{2+} transients in NRVMs (13, 17), and activation of IP_3Rs can robustly enhance the frequency of endogenous spontaneous Ca^{2+} transients in NRVMs, with or without gap junction channel formation.

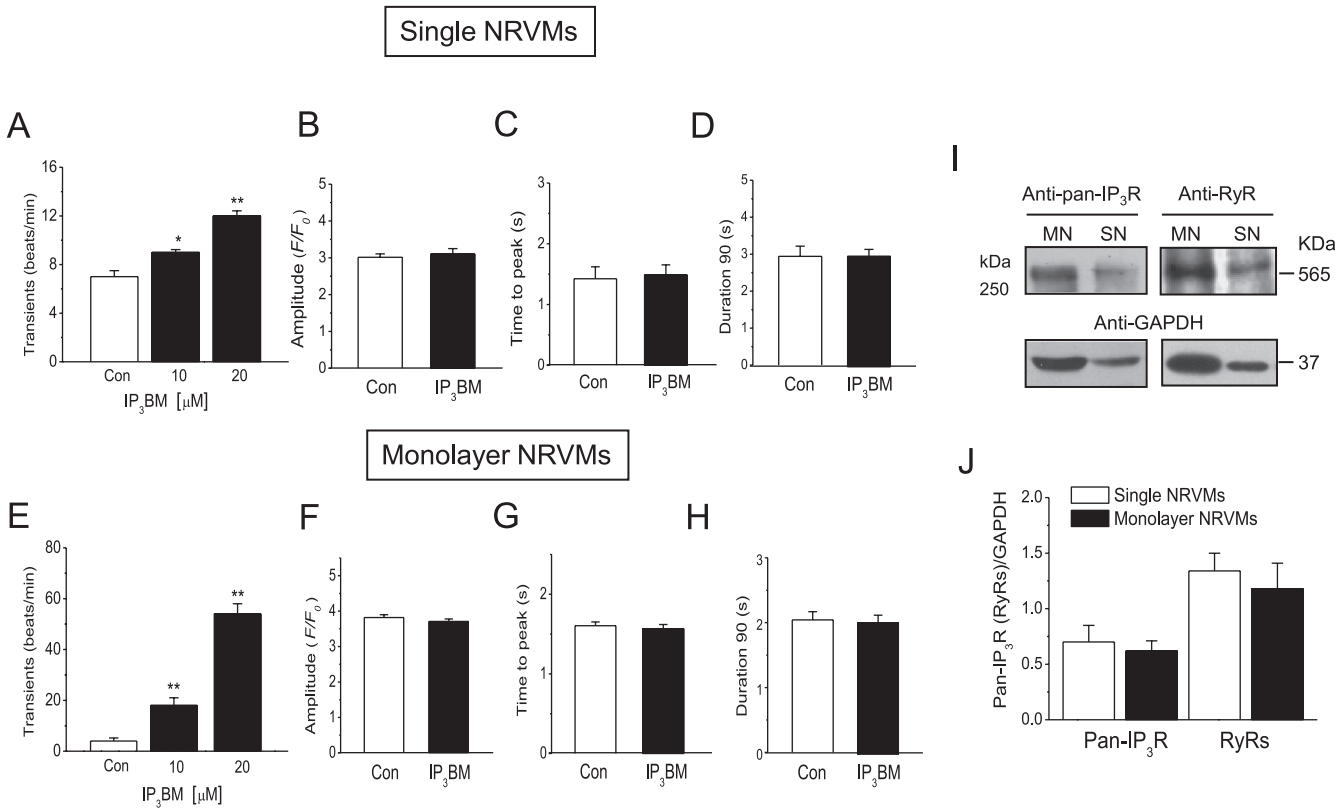

Fig. 2. Effect of IP_3R activation on spontaneous Ca^{2+} transients in single and monolayer cultured NRVMs. A – H) Dose–response effect of IP_3BM (for 6 min) on the Ca^{2+} transients in single (A – D) and monolayer (E – H) NRVMs, while the parameters of amplitude, time to peak, and duration 90 of the Ca^{2+} transients were unchanged (B – D and F – H). These data were obtained from 65 cells from 5 independent experiments for single NRVMs and 10 independent experiments for monolayer NRVMs. $^*P < 0.05$, $^{**}P < 0.01$ vs. Con for each panel. I) Expressions of IP_3Rs and RyRs were determined in single and monolayer NRVMs by western blotting using specific antibodies. Typical binding bands and the statistical data (J) after normalization show no significant change in the IP_3R and RyR levels between single and monolayer NRVMs. The statistical data of IP_3R and RyR expressions were normalized by the abundance of GAPDH in each group obtained from 3 independent experiments. MN and SN stand for monolayer and single NRVMs, respectively.

Effect of IP_3R interference on Ca^{2+} transients in single and monolayer cultured NRVMs

To further determine the role of IP_3Rs in the regulation of native Ca^{2+} activity in NRVMs, interferences of IP_3R1, IP_3R2, and IP_3R3 expression with shRNA specific against each isotype expression were performed (see Materials and Methods), as all three isoforms of IP_3Rs are expressed in neonatal hearts (7, 8, 10, 18). After transfection with all three IP_3R isotype shRNAs in combination for 48 h, NRVMs exhibited approximately 68% reduction in IP_3R abundance (Fig. 3: A and B) evaluated by western blotting, supporting an efficient knockdown of endogenous IP_3R expression. Although the interference of either IP_3R isoform did not show obvious effect on the spontaneous Ca^{2+} transients (data not shown), the knockdown of all the three isoforms of IP_3Rs significantly suppressed the rate and amplitude, but not the duration 90, of Ca^{2+} transients in both single and monolayer NRVMs (Fig. 3: C – J).

Effects of RyRs on Ca^{2+} transients in single and monolayer cultured NRVMs

RyRs are the critical Ca^{2+} effector in the control of myocardium Ca^{2+} transients and contraction. To compare it with the regulatory role of IP_3Rs, the effects of RyR inhibition were further determined on this cell model. Ryanodine at concentrations of 10, 30, and 100 μM and tetracaine at 0.3 and 0.5 mM were used to inhibit RyRs, respectively. As shown in Fig. 4, ryanodine and tetracaine (data not shown) dose-dependently attenuated the rate, amplitude, and duration 90 of the spontaneous Ca^{2+} oscillations, and an almost abolishment of Ca^{2+} transients was observed in cells treated with ryanodine at the concentration of 100 μM in both single and monolayer

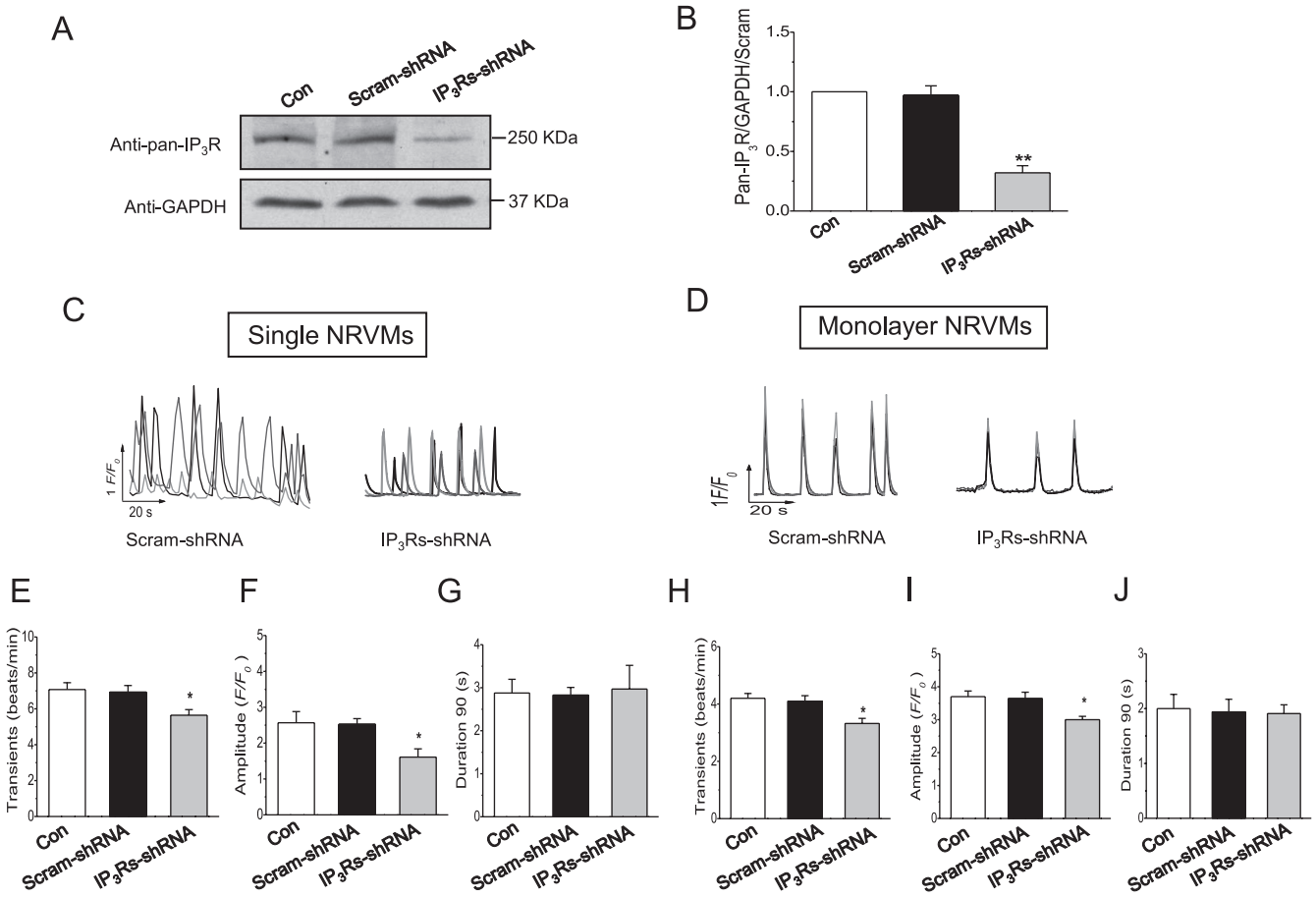

Fig. 3. Effect of IP_3R interference on Ca^{2+} transients in single and monolayer cultured NRVMs. A and B) NRVMs were transduced with adenovirus carrying all three IP_3R isoform shRNAs (90 m.o.i.) for 48 h (see Materials and Methods). The expression of IP_3Rs in NRVMs was determined by western blotting. The statistical data of IP_3Rs expression were normalized by the abundance of GAPDH in each group obtained from 5 independent experiments. $^{**}P < 0.01$ vs. Scram. C – J) Typical traces represent the spontaneous Ca^{2+} transients in single (C) and monolayer NRVMs (D), and the statistical data of the Ca^{2+} transient frequency, amplitude, and duration 90 in single (E – G) and monolayer (H – J) NRVMs as indicated were obtained from 57 cells from 5 independent determinations for single NRVMs and 12 independent experiment for monolayer NRVMs. $^{*}P < 0.05$ vs. Con for each panel, respectively.

NRVMs (Fig. 4: A – H). PE or IP_3BM could partially recover the Ca^{2+} transients after ryanodine treatment (8). Thus, these data support that RyRs are essential for the generation of global Ca^{2+} transients (3, 4, 19), while IP_3Rs, independent of RyRs, also participate in the regulation of Ca^{2+} signaling in cardiomyocytes.

Potential involvement of gap junctions in the regulatory effect of IP_3Rs on Ca^{2+} transients

It is well recognized that IP_3Rs distribute on the endoplasmic/sarcoplasmic reticulum and regulate the Ca^{2+} release process upon cell activation. Thus, one candidate mechanism underlying the regulatory effect of IP_3Rs is likely due to the interference with the Ca^{2+} mobilization. Additionally, other possible factors may also be involved; in particular that IP_3Rs have been found to localize to the discs in rat ventricles (14) and that gap junction uncouples do affect Ca^{2+} spiking significantly in ventricular myocytes (13). Thus, we finally determined whether IP_3Rs are co-localized with Cx43 (the predominant connexin in ventricular myocytes) in the gap junctions of NRVMs. Double-immunostaining of single and monolayer NRVMs with anti-pan-IP_3R and anti-Cx43 antibodies displayed that Cx43 is clustered at the sites of cell-to-cell appositions where it is co-localized with IP_3Rs (yellow color in Fig. 5B). Similarly, a detectable co-distribution of the two proteins on the cell membrane, presumably hemi-channels of gap junctions, was observed in single NRVMs (indicated with a white arrow in Fig. 5A); however, there were Cx43 proteins unassociated with IP_3Rs on the single NRVM membrane (indicated with yellow arrow in Fig. 5A). Therefore, this observa-

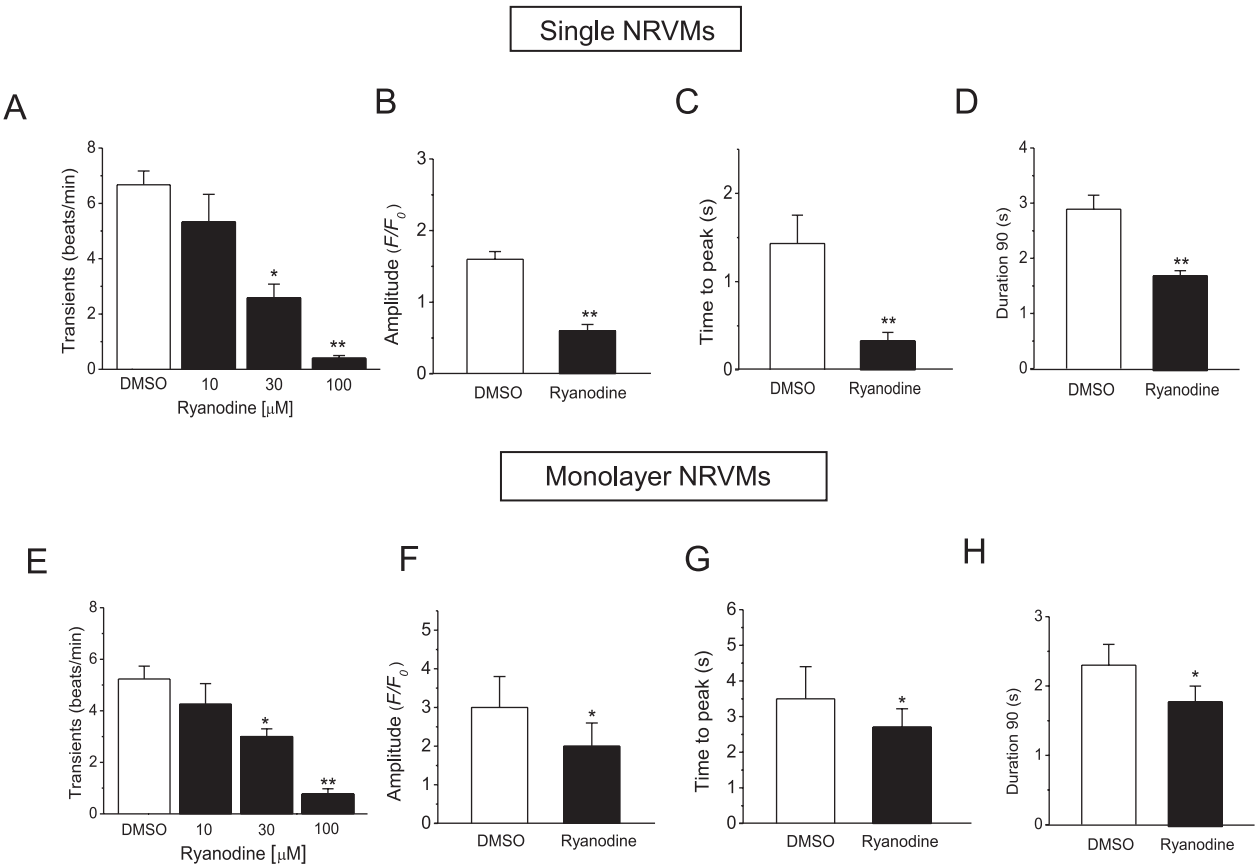

Fig. 4. Effects of RyR inhibition on Ca^{2+} transients in single and monolayer cultured NRVMs. A – H) Statistical data of Ca^{2+} transients prior to and 10 min after DMSO or ryanodine treatment in single (A – D) and monolayer (E – H) NRVMs loaded with the Ca^{2+} indicator fluo-4 as indicated. The dose-dependent effects of ryanodine on Ca^{2+} transients were obtained from 37 to 46 cells from 4 independent experiments for each panel in single NRVMs and 10 independent experiments for monolayer NRVMs. $*P < 0.05$, $**P < 0.01$ vs. DMSO for each panel, respectively.

tion is in agreement with the finding in adult ventricle (13, 17) and implies a potential interaction between Cx43 and IP_3Rs. Furthermore, we examined whether Cx43 expression was affected by IP_3R activation or interference using western blotting. The lysate was respectively collected from monolayer or single NRVMs that had been treated with PE (10 μM for 2 min), IP_3BM (20 μM for 6 min), scram-shRNA, or IP_3Rs-shRNA for 48 h and were labeled with specific anti-Cx43 antibody. No significant change in the Cx43 expression was found upon acute IP_3R activation, but the abundance of Cx43 expression was lowered in the monolayer NRVMs exposed to IP_3Rs-shRNA, but not in the single NRVMs, compared with that in scram-shRNA–treated NRVMs (Fig. 5: C – F). This observation may further reflect that IP_3Rs interact with Cx43 and somehow interfere with the functions of Cx43, but further study in detail is needed in this regard.

Discussion

The present study demonstrated that in both single and monolayer NRVMs, i) the rate of Ca^{2+} transients was significantly enhanced by activation of IP_3Rs with PE or IP_3BM (Figs. 1 and 2) and reduced by inhibition of IP_3Rs with 2-APB (13) or specific interference of IP_3R expression by shRNA (Fig. 3). In addition, the inhibition of RyRs with ryanodine did not affect the potentiating effect of PE (8), indicating that RyRs were not involved; ii) the amplitude of the native Ca^{2+} transient was also attenuated by the interference of IP_3Rs with 2-APB or IP_3R knockdown (Fig. 3); and iii) unlike the observations in ryanodine-treated NRVMs, which exhibited lowered rate, depressed amplitude, and shortened duration of Ca^{2+} transients (Fig. 4), the duration 90 in Ca^{2+} transients seemed not to be obviously affected by the treatment of IP_3R knockdown (Fig. 3). Therefore, these data support the proposal that the activation of IP_3Rs can somehow

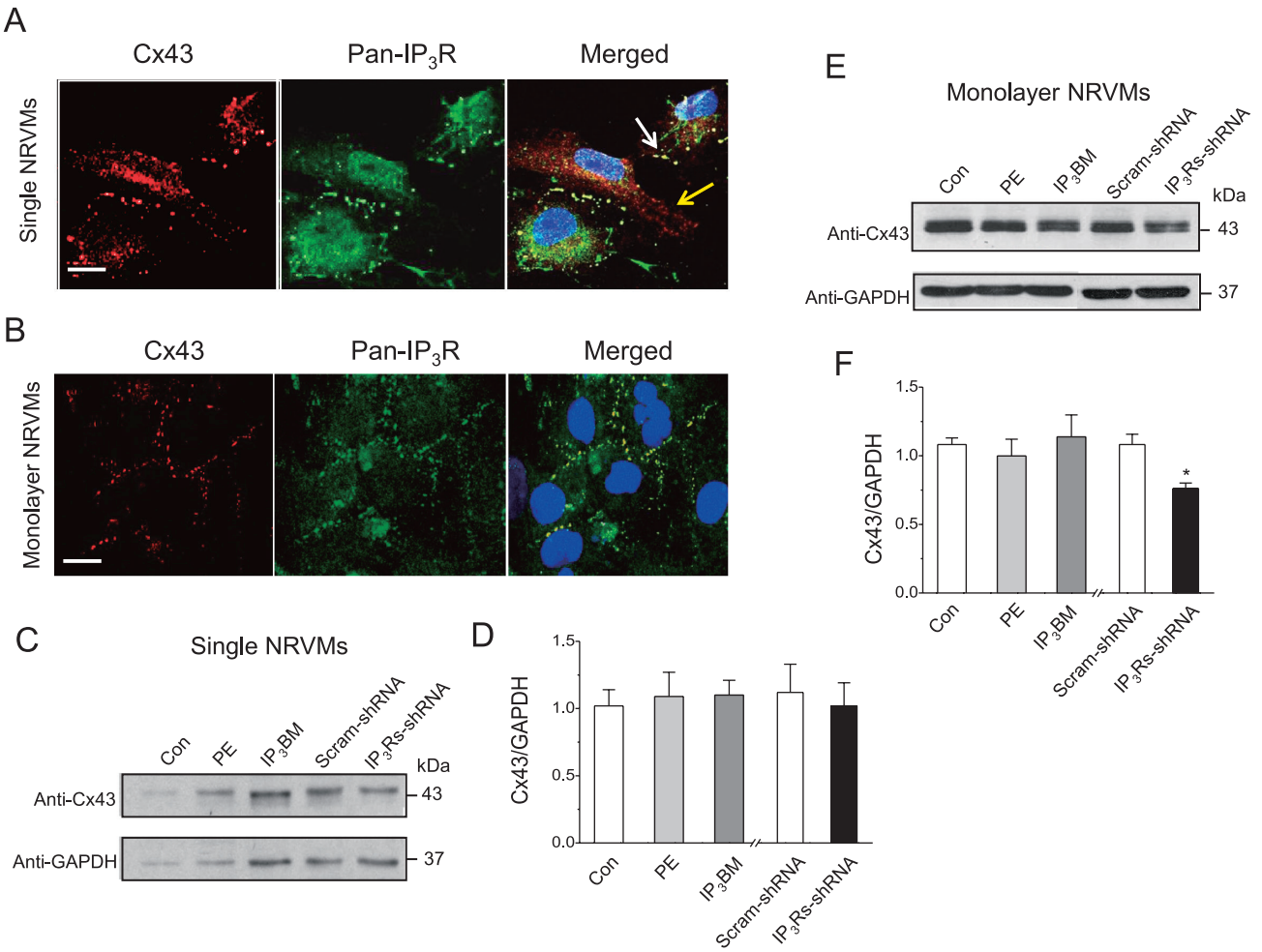

Fig. 5. Co-localization of IP_3Rs with Cx43 and the possible interaction between the two proteins. A and B) Cultured NRVMs were co-immunostained with anti-Cx43 and anti-pan-IP_3R antibodies and examined by a confocal microscope. The representative images show the distributions of Cx43 (red) and IP_3Rs (green), and their fractions (yellow) both in gap junctions of single (A) and monolayer (B) NRVMs. The nucleus was stained with Hoechst 33258 (1 μg/ml) and scale bar: 10 μm. The white and yellow arrows indicate co-distribution and un-association of Cx43 with IP_3Rs in single cells, respectively. C) Immunoblots of Cx43 after stimulated with PE, IP_3BM, Scram-shRNA, or IP_3Rs-shRNA in single NRVMs. D) The statistical data after normalization with the abundance of GAPDH in single NRVMs. E) Immunoblots of Cx43 after stimulating with PE, IP_3BM, Scram-shRNA, or IP_3Rs-shRNA in monolayer NRVMs. F) The statistical data after normalization with the abundance of GAPDH in monolayer NRVMs. The data in all panels are representatives of 4 independent experiments, and $^*P < 0.05$ vs. scram-NRVMs.

initiate spontaneous Ca^{2+} fluctuations (8, 11, 12, 18, 20), affects the native Ca^{2+} activities, and regulates the spontaneous Ca^{2+} signaling in resting NRVMs.

In heart, three isoforms of IP_3Rs are found in neonatal and adult ventricles. However, it is difficult to detect any functional differences between these isoforms because of the much low expression of each isotype in heart (7, 8, 18, 21). Thus we conducted knock-down of all three isoforms concomitantly in NRVMs and found a significant reduction in Ca^{2+} transient rate and amplitude in IP_3R-deficient NRVMs. Since no augmentation in the amplitude was found upon IP_3R activation with PE or IP_3BM in normal NRVMs (Figs. 1 and 2), whereas isoprenaline, an agonist of β-adrenergic receptor, did increase the amplitude of transients significantly (data not shown), the data suggest that IP_3R-mediated Ca^{2+} signal contributes to the constitution of basal Ca^{2+} transients, rather than promote Ca^{2+} release in each myocyte beat. Similar observations are also reported in other studies in adult ventricular myocytes, in which the amplitude of Ca^{2+} transient is not significantly changed, but premature beats appear upon exacerbated IP_3R activation (22, 23).

It is noticeable in this study that there are significant

differences in the basal Ca^{2+} and stimulated Ca^{2+} transient responses to stimulation between single and monolayer NRVMs. Firstly, the frequency of spontaneous basal Ca^{2+} transients in monolayer NRVMs was half of that in single NRVMs, and their amplitude and duration 90 were higher and shorter than those in single NRVMs (Fig. 1). Secondly, monolayer NRVMs were 5 to 10 times more sensitive to IP_3R activation than single NRVMs (Figs. 1 and 2). It has been well accepted that gap junction–mediated intercellular communication plays a crucial role in the electronic and metabolite spreading between adjacent cardiomyocytes (24, 25). Disordered gap junction exchange may cause cardiac arrhythmias and even myocytes apoptosis in ischemic heart (26, 27); and correction of gap junction coupling can ameliorate the premature heartbeats and ischemic necrosis (26). Additionally, in our previous study (13) and also others (17, 28), Cx43-mediated intercellular communication is involved in the regulation of Ca^{2+} oscillations, in particular modulating the rate of Ca^{2+} transients. Thus, the difference between single and monolayer NRVMs, may reflect an involvement of intrinsic gap junction communication in the modulation of cytosol Ca^{2+} activity in normal and stimulated cardiomyocytes, rather than potential difference in IP_3R expression in different densities (Fig. 2: I and J). To avoid this influence of gap junction on Ca^{2+} signaling in cardiomyocytes, single NRVMs should be more applicable to represent the actual effect of IP_3Rs or other signaling pathway on internal Ca^{2+} activities than monolayer NRVMs. Nevertheless, although with different potencies, the potentiating effects of IP_3R activation on Ca^{2+} transients were similar in single and monolayer NRVMs. In addition, the inhibitory effects of IP_3R knockdown with shRNA and IP_3R antagonism with 2-APB (13) reduced the endogenous Ca^{2+} transient rate and amplitude in both single and monolayer NRVMs. Therefore, these observations did reflect the entity of IP_3R action on the global Ca^{2+} signal in NRVMs.

Interestingly, the present study found that IP_3Rs are distributed in gap junctions of ventricular myocytes, consistent with the finding in rat ventricles (14). Additionally, they co-localized with Cx43 extensively in monolayer cells, but not so obviously in single cells (Fig. 5: A and B). However, no significant change was found in Cx43 expression upon IP_3R activation with PE or IP_3BM in both single and monolayer NRVMs (Fig. 5: C – F). The down-regulated Cx43 expression observed in IP_3R-knockdown monolayer NRVMs (Fig. 5: E and F) demonstrated a long-term effect of IP_3R interference with the Cx43 expression, as well as a support for the interaction between IP_3Rs and Cx43 in gap junctions. Moreover, this finding also provide a candidate explanation for why activation or interference of IP_3Rs can strikingly affect the rate of Ca^{2+} transients by their interfere with Cx43 functions, in addition to the impact on Ca^{2+} stores in cardiomyocytes. However, further study is needed to define how they are interacting and what signaling pathway is involved, especially for the acute effect of IP_3/IP_3R on the regulation of gap junction function.

This study also observed a minor difference in the effects of IP_3Rs and RyRs on the transient duration. Inhibition of RyRs accordingly inhibited the rates, amplitude, and duration of Ca^{2+} transients, whereas the interference of IP_3Rs did not affect the duration of Ca^{2+} transients, although both the transient rate and amplitude were attenuated. Even prolonged Ca^{2+} transients (duration 90 > 5 s) were found in 35.6% ± 2.3% NRVMs with IP_3R knockdown vs. 2.3% ± 0.3% in scrambled shRNA–treated NRVMs. However, it is not yet clear for this exceptional phenomenon and thus a further investigation is needed. Dysfunctional IP_3Rs have been implicated in the generation of arrhythmias, cardiac hypertrophy, and ischemia (29 – 31). In addition, IP_3Rs have been found to contribute to the heart beat through its distribution in and activation of sinoatrial node (10). This study also demonstrates a robust effect of IP_3Rs on the regulation of the native Ca^{2+} transients, in particular the frequency and the abundance of Ca^{2+} release in developing cardiomyocytes, suggesting additional effect of the IP_3/IP_3R signaling pathway on the spontaneous Ca^{2+} transients in ventricular myocytes.

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