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Isoprenaline enhances local Ca^{2+} release in cardiac myocytes[1]

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Key words

local calcium release; calcium signaling; cardiac myocytes; isoprenaline; confocal microscopy

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Abstract

Aim: Contraction of cardiac myocytes is controlled by the generation and amplification of intracellular Ca^{2+} signals. The key step of this process is the coupling between sarcolemma L-type Ca^{2+} channels (LCCs) and ryanodine receptors (RyRs) in the sarcoplasmic reticulum (SR). β-Adrenergic stimulation is an important regulatory mechanism for this coupling process. But the details underlied the global level, which require local Ca^{2+} release study are still unclear. The present study is to explore the effects of β-adrenergic stimulation on local Ca^{2+} release. **Methods:** Using confocal microscopy combined with loose-seal patch-clamp approaches, effects of isoprenaline (1 μmol·L^{-1}), a β-adrenergic agonist, on local SR Ca^{2+} release triggered by Ca^{2+} influx through LCCs in intact rat cardiac myocytes were investigated. **Results:** Isoprenaline increased the intensity of ensemble averaged local Ca^{2+} transients, the peak of which displayed a typical bell-shaped voltage-dependence over the membrane voltages ranging from ~-40mV to ~+35mV. Further analysis showed that this enhancement could be explained by the increased coupling fidelity (which refers the increased probability of RyRs activation upon depolarization), and the increased amplitude of evoked Ca^{2+} sparks (due to more Ca^{2+} releases through local RyRs). In addition, isoprenaline decreased the first latency, which displayed a typical "U"-shaped voltage-dependence, showing the available acceleration and synchronization of β-adrenergic stimulation on intracellular calcium release. **Conclusions:** Isoprenaline enhances local Ca^{2+} release in cardiac myocytes. These results underscore the importance of regulation of β-adrenergic stimulation on local intermolecular signals between LCCs and RyRs in heart cells.

Introduction

Contraction of cardiac myocytes is controlled by the generation and amplification of intracellular Ca^{2+} signals, which are composed of the elementary Ca^{2+} release events or Ca^{2+} sparks, emitting from the ryanodine receptors (RyRs) in the sarcoplasmic reticulum (SR)[1,2]. This process is so called the excitation-contraction (EC) coupling[3,4]. Upon depolarization, RyRs could be activated by Ca^{2+} influx via sarcolemma L-type Ca^{2+} channels (LCCs). Physiologically, temporal and spatial summation of Ca^{2+} sparks through RyRs from SR are synchronized by the trigger Ca^{2+} influx through LCCs ($I_{Ca,L}$). The mechanism for this process is termed Ca^{2+}-induced Ca^{2+} release (CICR)[3,4]. Activation of LCCs and RyRs are with a specific probability that may depend on some complicated regulatory mechanisms (eg, β-adrenergic stimulation) or diseased states (eg, heart failure)[2,5,6].

β-Adrenergic stimulation is an important physiological inotropic pathway which has been well characterized in cardiac myocytes[2,4,7,8]. Generally, in heart physiology, β-adrenergic stimulation increases muscle contractions (inotropy) and accelerates relaxation (lusitropy). First, stimulation of β-adrenergic receptors by agonists activates a GTP-binding protein (G_S), which leads to activation of adenylate cyclase (AC). And then, the level of intracellular cyclic adenylic acid (cAMP) goes up, which in turn activates protein kinase A

(PKA). PKA phosphorylates several proteins that related to excitation-contraction coupling (eg, phospholamban, LCCs, RyRs, troponin I and myosin binding protein C, and *etc*). Many studies showed that β-adrenergic stimulation may enhance SR Ca^{2+} release by increasing the $I_{Ca,L}$ trigger (due to phosphorylation of LCCs), or by increasing the SR Ca^{2+} load (due to phosphorylation of phospholamban), or by enhancing the cross signaling between LCCs and RyRs (due to phosphorylation of LCCs and RyRs). However, the precise mechanisms responsible for such alterations in the intracellular local Ca^{2+} release remain incompletely understood, partly due to lack of direct investigating approaches[9].

In the present study, a new proper method, confocal microscopic imaging combined with loose-seal patch-clamp approaches (loose-patch method)[9,10] was employed to investigate effects of isoprenaline (1 μmol·L^{-1}), a β-adrenergic agonist, on local SR Ca^{2+} release triggered by the Ca^{2+} influx through LCCs in intact rat cardiac myocytes.

Materials and methods

Single heart cell preparation Enzymatically isolated ventricular myocytes from adult Sprague-Dawley rats (age, 2–3 months; weight, 225–300 g) were loaded with Ca^{2+} indicator Fluo-4-AM (15 μmol·L^{-1}) (Molecular Probes, Eugene, OR) for 5−8 min, followed by a 10-min rest allowing for de-esterfication of the indicator, as described previously[9]. The criteria for cell selection included rod shape, clear striation and clean cell surface, and lack of spontaneous contractions during a 1-min observation period.

Solution and isoprenaline application Isoprenaline (ISO) was freshly made (about 2 h before use) and perfused with extracellular fluid to act on cardiac myocytes 5 min before experiments and reached a final concentration of 1 μmol·L^{-1}. The extracellular and patch pipette filling solution contained (in mmol·L^{-1}): 137 NaCl, 1 $CaCl_2$, 4.9 KCl, 1 $MgCl_2$, 1.2 NaH_2PO_4, 15 glucose, and 20 HEPES (pH 7.4, adjusted with NaOH).

Loose-seal patch-clamp Cell-attached patch-clamping was established using axopatch 200B amplifier (Axon Instruments, Foster City, CA) in loose-seal configuration, as described previously[9,10]. A glass pipette (3–5 MΩ, <1 μm at the tip) was gently pressed onto the selected cell surface to form a low resistance seal (20–40 MΩ). The patch membrane voltage (or potential) was determined according to the equation of V_{PM}= RP-*V*com·Rs/(Rs+Rp), where V_{PM} refers to the patch membrane voltage, RP the resting potentials (approximately -80 mV), *V*com the command voltage applied, Rs and Rp the seal resistance and pipette resistance, respectively.

Confocal Ca^{2+} imaging Ca^{2+} images were acquired by using a Zeiss LSM510 confocal microscope equipped with an argon laser (488 nm) and a 40×, 1.3 numerical aperture, oil-immersion objective, at sampling rates of 0.77 ms per line and 45 nm per pixel. Using loose-patch method, population of in-focus Ca^{2+} sparks could be evoked by repeated patch depolarization, with an interval of 6 s between two consecutive confocal microscopic images in line-scan mode. All experiments were performed at room temperature (23–25 °C).

Data analysis and statistics Ca^{2+} spark detection algorithm was almost the same as that described previously[11], with some minor modifications. Computer programs for the spark detection and measurement were coded in Interactive Data Language (IDL, Research Systems, Boulder, CO). Ca^{2+} spark amplitudes were measured as $\Delta R=\Delta F/F_0$, where *F* refers to the present Fluo signal intensity, F_0 the background Fluo signal intensity, and $\Delta F/F_0$ the alteration of F/F_0. Data were expressed as mean±SEM, if not otherwise specified. Ensemble averaged single-couplon Ca^{2+} transients images were guided by the onsets of the depolarization pulse (Figure 1), while averaged line-scan images of Ca^{2+} sparks by the peak position of each spark (Figure 4). In this study, single-couplon refers to one elementary Ca^{2+} release unit, including one or few LCCs and some coupled RyRs. The significance of difference between means or ratios was determined, when appropriate, by using the Student *t* test or the nonparametric Kruskal-Wallis test. $P<0.05$ was considered statistically significant.

Results

Effect of isoprenaline on local intracellular Ca^{2+} release revealed by ensemble averaged single-couplon Ca^{2+} transients To have a overall look at the effect of isoprenaline (ISO, 1 μmol·L^{-1}) on local intracellular Ca^{2+} release in cardiac myocytes, depolarization pulse with a duration of 100 ms were applied under loose-seal patch-clamp configuration and confocal line-scan Ca^{2+} images were obtained from 62 patches (27 patches under the control and 35 patches with ISO treatment). Pixel-to-pixel averaging of confocal line-scan images guided by the onsets of the depolarization pulse was performed on all runs grouped by membrane potentials, with an increment of 15 mV (Figure 1A and Figure 1B). These are called the images of ensemble averaged single-couplon Ca^{2+} transients (Figure 1A left and Figure 1B left). At all membrane potential levels (ranging from approximately approximately -40 mV to approximately +35 mV), ensemble averaged single-couplon Ca^{2+} transients triggered by the depolarization pulse after ISO treatment were much stronger than that triggered under control condition (Figure 1A and Figure 1B) and displayed a typical bell-shaped voltage-dependence

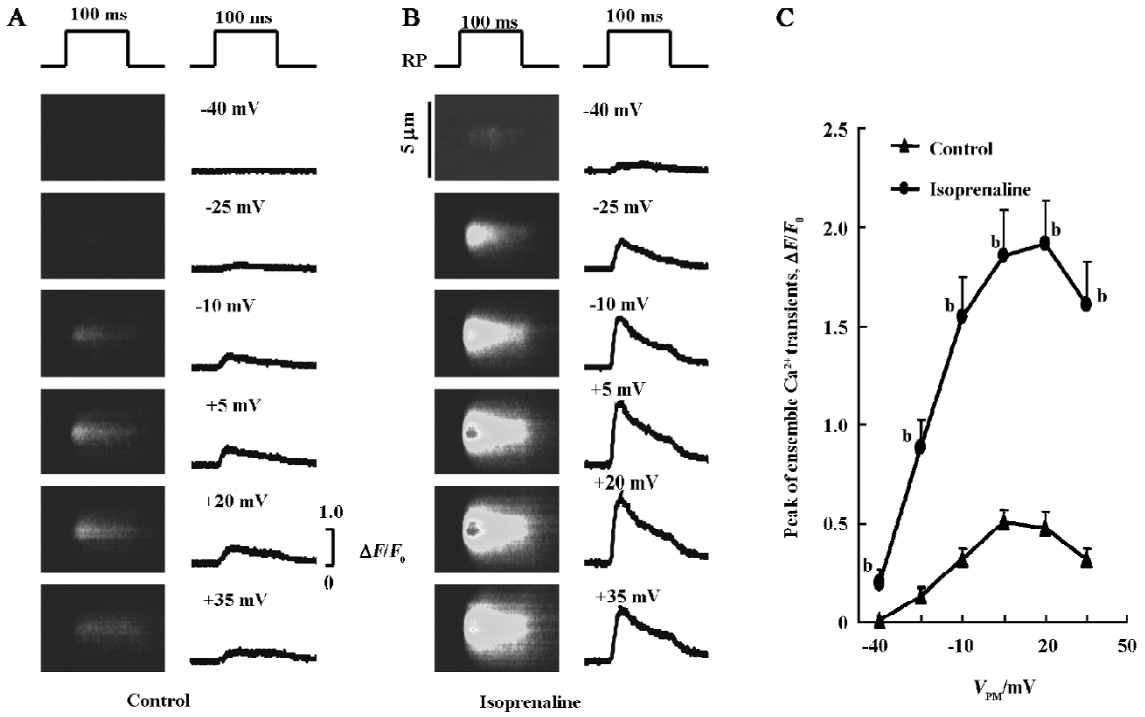

Figure 1. Effect of isoprenaline on ensemble averaged single-couplon Ca^{2+} transients. (A) Reuslts in control condition. Left, Spatiotemporal visualization of ensemble averaged single-couplon Ca^{2+} transients at six representative voltages. Pixel-to-pixel averaging of confocal images was performed on all runs grouped in 15-mV increments. Total runs for each group are from 29 to 44. Right, The line plots show time courses of the averaged local evoked Ca^{2+} transients. RP, resting potential. Sym $\Delta F/F_0$, refers to the alteration of Fluo-4 signal ratio to the background Fluo-4 signal. (B) Results after isoprenaline (1 μmol·L^{-1}). Total runs for each group are from 43 to 58. (C) Effect of isoprenaline on the peak amplitudes of ensemble averaged single-couplon Ca^{2+} transients. Con, peak of the averaged Ca^{2+} transients under the control condition. ISO, peak of the averaged Ca^{2+} transients under the isoprenaline condition. $^bP<0.05$ *vs* control.

(Figure 1C). These results showed that generally ISO enhanced the local intracellular Ca^{2+} release ($P<0.05$).

Effect of ISO on coupling fidelity Generally, enhancement of ensemble averaged single-couplon Ca^{2+} transients could be the result of the increased coupling fidelity and/or the increased amplitude of local Ca^{2+} release, both of which could be tested in the present study. Coupling fidelity of local Ca^{2+} release (including Ca^{2+} sparks) was analyzed first. In this study, coupling fidelity (δ) was determined by the number of active runs over the corresponding total number of all runs (including active runs and silent runs). Active runs refers to those images that recorded the triggered single-couplon Ca^{2+} release (including Ca^{2+} sparks). The peak of $\Delta F/F_0$ for an active run is at least 0.25 (Figure 4A). For instance, under the control condition, when V_{PM} =approximately 5 mV, total runs of 29 were acquired and 27 of them were active runs. So the coupling fidelity is 0.93, meaning that the probability for the Ca^{2+} influx through LCCs to successfully trigger RyRs activation is 93%. Results showed that at all membrane potential levels (ranging from ~-40 mV to ~+35 mV), coupling fidelity increased after ISO treatment (Figure 2) and displayed a left half bell-shaped voltage-dependence. These results explain one possible mechanism for enhancement of local intracellular Ca^{2+} release by ISO: to trigger more elementary Ca^{2+} release events. In other words, local Ca^{2+} release is easier to be activated with β-adrenergic stimulation.

Effect of ISO on averaged Ca^{2+} spark amplitude Next, effect of ISO on averaged Ca^{2+} spark amplitude was considered. Since the solitary Ca^{2+} sparks were difficult to separate from each other when the plasmic membrane was depolarized to higher voltage level (eg >10 mV), only those solitary Ca^{2+} sparks that were triggered around -30 mV or 0 mV were taken into account. Pixel-to-pixel averaging of confocal line-scan images of solitary Ca^{2+} sparks were guided by the peak position of each spark (Figure 3). Results showed that the evoked averaged Ca^{2+} sparks were stronger in ISO treatment condition than in control condition, at either ap-

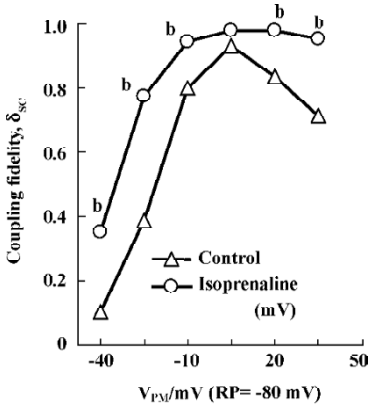

Figure 2. Effect of isoprenaline on single-couplon excitation-contraction coupling fidelity (δ_{SC}). δ_{SC} is shown as a function of patch membrane voltage (V_{PM}). δ_{SC} were determined by the numbers of active runs over the total numbers of runs at different voltage group in 15-mV increments. $^bP<0.05$ *vs* control.

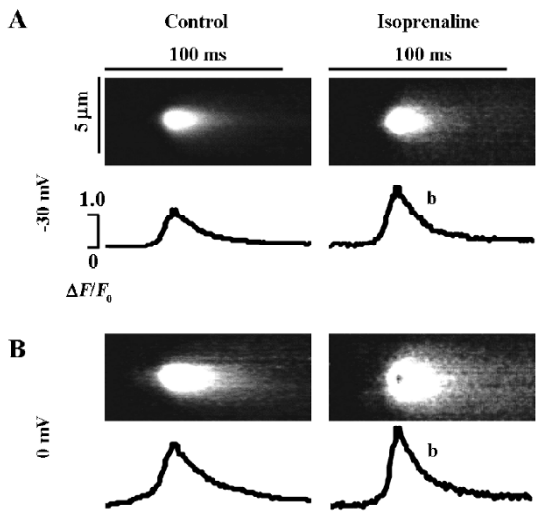

Figure 3. Effect of isoprenaline on averaged sparks at different voltages. (A) Averaged spark at ~-30 mV. From top to bottom: averaged line-scan images of sparks at peak, time courses of the corresponding line-scan images (spatially averaged over 1.1-μm width); left: averaged spark under the control condition; right: averaged spark after isoprenaline (1 μmol·L^{-1}) treatment. (B) Averaged spark at ~0 mV. $^bP<0.05$ *vs* control.

proximately-30mV or approximately 0 mV (Figure 3). That is, β-adrenergic stimulation enhances the intensity of single elementary Ca^{2+} release event.

Effect of ISO on the first latency of couplon activation (L_{SC}) In addition, the first latency of couplon activation (L_{SC}) was investigated, too. The first latency of couplon activation refers to the duration from the onset of the depolarization pulse to the onset of the first Ca^{2+} spark that is triggered by this depolarization pulse (Figure 4A). The results showed that the first latency were shorter in ISO treatment condition than in control condition ($P<0.05$) (Figure 4B) and displayed

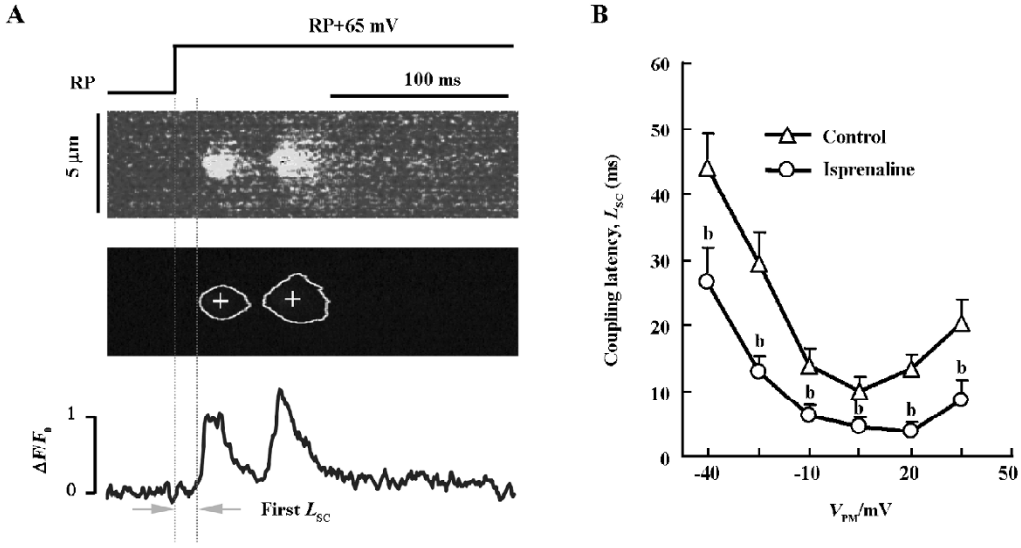

Figure 4. Effect of isoprenaline on the first latency of couplon activation (L_{SC}). (A) A typical case to show the first latency measurement. From top to bottom: the voltage protocol, line-scan image of the evoked spark, its contour plot at $\Delta F/F_0$=0.25 and its mass center (marked by the cross), and time course of local Ca^{2+} transient (spatially averaged over 1.1-μm width). The mass center coordinates (X, T) were determined by X=($\int\int x\Delta F dx dt$)/($\int\int \Delta F dx dt$) and T= ($\int\int t\Delta F dx dt$) /($\int\int \Delta F dx dt$). The mass center coordinates were used to determine whether two or more Ca^{2+} sparks in one image were activated from the same single couplon (see Ref 9). (B) L_{SC} is shown as a function of patch membrane voltage (V_{PM}). $^bP<0.05$ *vs* control.

a typical "U"-shaped voltage-dependence over membrane potentials ranging from approximately -40 mV to approximately +35 mV. This suggests that after ISO treatment (ie β-adrenergic stimulation), single Ca^{2+} release unit (or single-couplon) is more sensitive to be activated.

Discussion

Physiologically sympathetic nerve regulates heart muscles through β-adrenergic receptors to generate positive inotropy (increasing contractility), positive chronotropy (increasing heart rate), positive dromotropy (increasing conduction velocity) and lusitropy (relaxation acceleration)[12,13]. Inotropy and lusitropy are tightly related to the process of CICR. Inotropic effect is mediated by the combination of increased Ca^{2+} influx and greater availability of SR Ca^{2+}[2,4]. And lusitropic effect is mediated by phosphorylation of phospholamban and troponin I, which speed up SR Ca^{2+} reuptake and dissociation of Ca^{2+} from the myofilaments[2,4]. When CICR is activated in a synchronized way on a global cell level, the Ca^{2+} signals are predominantly governed by SR Ca^{2+} content, which defines the amplitude of the resulting Ca^{2+} transients[2,14]. However, when CICR is activated on a local level, the Ca^{2+} signals behave in a dramatically different way, which cannot be explained only by global changes in SR Ca^{2+} content[2]. Obviously, local Ca^{2+} release depends somewhat on the SR Ca^{2+} load. But since local SR Ca^{2+} release only occurs from one or a few functional units of the SR, local SR Ca^{2+} content depletion by local Ca^{2+} release can be refilled instantly from neighbouring SR network [2,15]. Enhancement of global Ca^{2+} release by ISO has been well addressed in some previous studies[2,4,7]. However, details of local Ca^{2+} release are still unclear. In the present study, confocal microscopic imaging combined with loose-seal patch-clamp approaches is a proper way to explore local intracellular Ca^{2+} release directly [9,10].

Enhancement of ensemble averaged single-couplon Ca^{2+} transients by ISO is consistent with previous works at global level As stated in the results, ensemble averaged single-couplon Ca^{2+} transients were increased by ISO and their peak amplitudes displayed a typical bell-shaped voltage-dependence, just like the relationship between L-type Ca^{2+} currents ($I_{Ca,L}$) and membrane voltages[7]. This confirmed the local Ca^{2+} signals were triggered by Ca^{2+} influx through L-type Ca^{2+} channels. These results were consistent with the observations that β-adrenergic stimulation can greatly enhances Ca^{2+} transients amplitude on global level[2,4,7].

Enhancement of local Ca^{2+} release by ISO could be explained by the increased coupling fidelity and the increased amplitude of evoked Ca^{2+} sparks Results showed that both coupling fidelity of single-couplon (or single Ca^{2+} release unit) and evoked Ca^{2+} spark amplitude were increased significantly by ISO treatment. This suggests that β-adrenergic stimulation makes more single-couplons of Ca^{2+} release ready to be activated; and in each activation, more Ca^{2+} releases through SR Ca^{2+} release channels (or RyRs) from SR. The latter may be due to increased RyRs open probability (one result of phosphorylation of RyRs by β-adrenergic stimulation)[16–18] and increased SR Ca^{2+} load (the result of phosphorylation of phospholamban, a relief to inhibit the SR Ca^{2+} pump uptaking) [2,4,19].

Significance of the shortened first latency of local Ca^{2+} release by ISO The present results also showed that ISO could shorten the first latency of local Ca^{2+} release and displayed a typical "U"-shaped voltage-dependence. First latency of local Ca^{2+} release indicates the shortest time to activate an elementary Ca^{2+} release. The results suggest that β-adrenergic stimulation accelerates local Ca^{2+} release activation and thus synchronizes intracellular Ca^{2+} release on global level[7]. This may be due to the bigger triggered Ca^{2+} influx (the result of phosphorylation of LCCs) and increased sensitivity of Ca^{2+} release unit to Ca^{2+} (the result of phosphorylation of RyRs and phospholamban) [2,4,7,20].

In conclusion, using confocal microscopic imaging combined with loose-seal patch-clamp approaches, effects of isoprenaline (ISO, 1 $\mu mol \cdot L^{-1}$) on local SR Ca^{2+} release triggered by Ca^{2+} influx through LCCs in intact rat cardiac myocytes were investigated in the present study. ISO, acting as a β-adrenergic agonist, increased the intensity of ensemble averaged local Ca^{2+} transients and displayed a typical bell-shaped voltage-dependence. This enhancement could be explained by two aspects of data: the increased coupling fidelity (which refers the probability of RyRs activation upon depolarization), and the increased amplitude of evoked Ca^{2+} spark (due to more Ca^{2+} releases through local RyR activation from SR). In addition, ISO could decreased the first latency of local evoked Ca^{2+} signals and displayed a typical "U"-shaped voltage-dependence. All these data were consistent with some previous studies on global levels. Furthermore, confocal microscopic imaging combined with loose-seal patch-clamp approaches reveals more subcellular details, especially the Ca^{2+} spark amplitude and the first latency of local Ca^{2+} release, which could not be unraveled by other methods previously. All these results underscore the importance of regulation (eg phosphorylation) of β-adrenergic stimulation on LCCs and RyRs or some other related proteins to enhance local Ca^{2+} signals [2,4]. This may also provide new therapeutic avenues to recover impaired Ca^{2+}

signaling during cardiac disease [5,6,12].

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