Dual mode of action of IP$_3$-dependent SR-Ca$^{2+}$ release on local and global SR-Ca$^{2+}$ release in ventricular cardiomyocytes$^{\#}$

Caroline Egger$^{a,b,1}$, Miguel Fernandez-Tenorio$^{a,1}$, Joaquim Blanch$^a$, Radoslav Janicek$^a$, Marcel Egger$^{b,2}$

$^a$ Department of Physiology, University of Bern, Buehlplatz 5, CH 3012 Bern, Switzerland
$^b$ Department of Emergency Medicine (Notfallzentrum) Inselspital - University of Bern, Freiburgstrasse 10, CH 3010 Bern, Switzerland

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**ABSTRACT**

In heart muscle, the physiological function of IP$_3$-induced Ca$^{2+}$ release (IP$_3$ICR) from the sarcoplasmic reticulum (SR) is still the subject of intense study. A role of IP$_3$ICR may reside in modulating Ca$^{2+}$-dependent cardiac arrhythmogenicity. Here we observe the propensity of spontaneous intracellular Ca$^{2+}$ waves (SCaW) driven by Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) in ventricular myocytes as a correlate of arrhythmogenicity on the organ level. We observe a dual mode of action of IP$_3$ICR on SCaW generation in an IP$_3$R overexpression model. This model shows a mild cardiac phenotype and mimics pathophysiological conditions of increased IP$_3$R activity. In this model, IP$_3$ICR was able to increase or decrease the occurrence of SCaW depending on global Ca$^{2+}$ activity. This IP$_3$ICR-based regulatory mechanism can operate in two “modes” depending on the intracellular CICR activity and efficiency (e.g. SCaW and/or local Ryanodine Receptor (RyR) Ca$^{2+}$ release events, respectively): a) in a mode that augments the CICR mechanism at the cellular level, resulting in improved excitation-contraction coupling (ECC) and ultimately better contraction of the myocardium, and b) in a protective mode in which the CICR activity is curtailed to prevent the occurrence of Ca$^{2+}$ waves at the cellular level and thus reduce the probability of arrhythmogenicity at the organ level.

**1. Introduction**

The presence of 1,4,5-trisphosphate-induced intracellular Ca$^{2+}$ release (IP$_3$ICR) has been established in cardiomyocytes for some time [1] however its regulatory function in cardiac excitation-contraction coupling is still subject of ongoing debate.

In contrast to Ca$^{2+}$-induced Ca$^{2+}$ release, IP$_3$ICR requires the intracellular synthesis of IP$_3$ triggered by G-protein-coupled receptor (GPCR) activation which is in turn induced by hormone binding. Although IP$_3$ICR may not strongly contribute to the global Ca$^{2+}$ transient amplitude in ventricular myocytes, a more complex role of modulating CICR under conditions of cardiac pathophysiological remodeling has been suggested. Several cardiac pathologies, in particular Ca$^{2+}$-dependent pro-arrhythmogenicity (e.g. delayed after depolarizations) [1–3] have been associated with a functional up-regulation of IP$_3$ receptors (IP$_3$Rs). Moreover, evidence indicates that G protein-coupled receptor (GPCR) activation is time dependent and that IP$_3$ICR may have a different impact in the short versus the long term. In the short term, binding of Ca$^{2+}$ to RyR could lead to increased open probability of the receptor (Ca$^{2+}$-dependent sensitization) in the presence of trigger Ca$^{2+}$ provided by the opening of the L-type Ca$^{2+}$ channel, thus facilitating SR-Ca$^{2+}$ release; in the long term, depleted SR-Ca$^{2+}$ stores could lead to an opposite, a Ca$^{2+}$-dependent desensitization of the RyR and thus to reduced RyR2 open probabilities. Consequently, in the latter respect, the CICR chain reaction would be impaired [4].

In this study we were able to provide evidence supporting this dual-action hypothesis by using an IP$_3$R overexpressing mouse model. The timing / duration of IP$_3$ICR interaction with the CICR machinery plays fundamentally a modulatory role for global SR-Ca$^{2+}$ release events, e.g. Ca$^{2+}$ wave appearance and/or Ca$^{2+}$ wave speed. The dual regulatory mechanism presented in this study is a new facet of intracellular Ca$^{2+}$ homeostasis that has not been described before, but which has a

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$^\#$ A brief statement explaining the importance of the results and why rapid publication is justified is given in the accompanying cover letter.

$^*$ Corresponding author.

E-mail address: marcel.egger@unibe.ch (M. Egger).

$^1$ Shared co-first authorship. C. Egger and M. Fernandez-Tenorio contributed equally to the present manuscript.

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significant impact on the direction of the remodeling process and should be considered. In this manner the present study makes a contribution in the field for the understanding of global ECC remodeling in which IP$_3$-Ca$^{2+}$ release plays a significant role.

2. Material and methods

All experiments were approved by the State Veterinary Office of Bern, Switzerland, according to Swiss Federal Animal Protection Law and performed at room temperature (22 °C). Ventricular myocytes were isolated from mice specifically overexpressing IP$_3$R type 2 in cardiac tissue [5]. More details about UV flash photolysis of caged IP$_3$ in saponin-permeabilized and intact ventricular myocytes are given in the supplementary material.

Each dot in a figure represents a measurement from an individual cell. The number of animals (N) and cells (n) are given in the figure legends. Statistical analysis was performed by fitting a linear mixed-effects model with 1 level (isolation) to data and pairwise comparison with Benjamini–Hochberg correction was used to test significant differences among individual groups.

3. Results and discussion

We conducted experiments with permeabilized cardiomyocytes isolated from transgenic (TG) mice overexpressing (functional) IP$_3$R. This TG model was originally introduced by Nakayama et al. in 2010 [7] and is one of the few available models to study the role of IP$_3$ICR in Ca$^{2+}$ signaling in cardiomyocytes in which the relative ratio of IP$_3$R and RyRs is significantly increased, mimicking some of the remodeling observed in several cardiac pathologies. It is characterized by a mild phenotype with an upregulation of functionally expressed IP$_3$R by about 13 times in ventricles [6] and was successfully used in recent studies [6,7].

The appearance of local SR Ca$^{2+}$ release events and Ca$^{2+}$ waves in response to acute global intracellular IP$_3$ concentration jumps were studied by UV-flash photolysis of caged IP$_3$. Uncaging of caged IP$_3$ will increase the global intracellular IP$_3$ concentration in the entire cell in a rapid (“acute”) and homogeneous fashion within ~2 ms. To mimic a “chronic” IP$_3$ response we alternatively used ET-1 to activate GPCR-induced intracellular IP$_3$ synthesis, which increases intracellular IP$_3$ levels orders of magnitude more slowly than UV photorelease. However, also protein kinase C (PKC) activation may be part of ET-1-GPCR pathway stimulation. Although PKC affects various intracellular targets, we demonstrated that ET-1-triggered arrhythmogenic responses could be suppressed by blocking IP3R2. This indicates that IP3ICR is the main mechanism for arrhythmic responses and that other PKC phosphorylation effects are minor in this setting.

In a series of control experiments, IP$_3$ photorelease triggered an increase in appearance of local Ca$^{2+}$ release events (p = 0.0010) (Supplemental Fig. 1). This acute response supports the idea of a boosting effect of IP$_3$ICR in triggering and shaping local Ca$^{2+}$ events. A more detailed description is given in the supplements. The set of control experiments also indicates that the majority and surplus IP$_3$ triggered Ca$^{2+}$-events have the spatio-temporal characteristics of Ca$^{2+}$ sparks. In addition to the observable Ca$^{2+}$-events (e.g. Ca$^{2+}$ sparks, Ca$^{2+}$ puffs) based on synchronized openings of clustered Ca$^{2+}$ release channels, eventless SR-Ca$^{2+}$ release may occur. This behavior is possible due to individual or non-functional clustered IP$_3$Rs openings [8].

Next, we focused on Ca$^{2+}$ waves affected by IP$_3$ICR in ventricular myocytes. The underlying mechanisms of spontaneous Ca$^{2+}$ waves are CICR and their occurrence under physiological conditions (1.8 mmol/l [Ca$^{2+}$]_o) is rather low. Pro-arrhythmogenicity on the organ level is connected with the occurrence of Ca$^{2+}$ waves on the cellular level.

To investigate a potential modulatory role of IP$_3$ICR on Ca$^{2+}$ waves the protocol shown in Fig. 1A was applied to resting myocytes (Fig. 1B, C). Since the SR-Ca$^{2+}$ content represents a regulatory mechanism for CICR [9,10] intact ventricular cardiomyocytes were externally paced to keep the SR- Ca$^{2+}$ content at comparable levels (Fig. 1A) and loaded with an AM form of caged IP$_3$. Fig. 1B shows examples of spontaneous Ca$^{2+}$ waves before and after photorelease of IP$_3$. After IP$_3$ photorelease Ca$^{2+}$ wave speed was promptly accelerated from 74.20 [68.39, 80.02] to

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Fig. 1. Spontaneous intracellular Ca$^{2+}$ waves after UV-flash photolysis of caged IP$_3$ in TG intact ventricular myocytes. (A) Protocol applied to intact cardiomyocytes to examine SCaW parameters. After 30 s of 2 Hz external field stimulation SCaWs were detected for 30 s in control conditions or with UV flash applied 1 s after last triggered Ca$^{2+}$ transient. SR-Ca$^{2+}$ load was assessed by 10 mmol/l caffeine. (B) Line scans showing SCaW before and after photolytic IP$_3$ release. (C) The Ca$^{2+}$ wave speed significantly increases after IP$_3$ liberation by UV-flash. $N = 6$, $N_c = 20$, p-values of <0.05 are indicated by *.
84.81 [74.99, 94.64] μm/s (mean [95% CI], \( p = 0.0150 \), Fig. 1 C) supporting the hypothesis of a boosting function of IP\(_3\)ICR for CICR in intact cardiomyocytes. In addition, no significant changes of Ca\(^{2+}\) wave amplitude, latency or frequency were detected (Supplemental Fig. 2).

In Fig. 2 an essentially identical experimental protocol was applied, now based on direct GPCR activation with the vasoconstrictor peptide endothelin-1 (ET-1) in order to generate IP\(_3\) intracellularly in intact cardiomyocytes. This also mimics a more “chronic” IP\(_3\) effect established in a time range of seconds to minutes.

In contrast to the acute IP\(_3\) photorelease response, here the Ca\(^{2+}\) wave speed was unchanged. In addition to Ca\(^{2+}\) wave frequency, we also analyzed the latency to the appearance of the first Ca\(^{2+}\) waves after application of the SR-Ca\(^{2+}\) loading protocol. Unexpectedly, in the presence of ET-1, the cells appeared to respond differently, with some cells responding with a spontaneous Ca\(^{2+}\) wave frequency increase, while others appeared to show lower wave activity. Analysis of the control situation without ET-1 showed the following correlation with the ET-1 response: (1) responders with an initial low spontaneous Ca\(^{2+}\) wave activity showed a tendency to increase the frequency of Ca\(^{2+}\) waves in the presence of ET-1 stimulation and (2) responders with an initial high spontaneous Ca\(^{2+}\) wave activity showed the opposite behavior, a tendency to decrease the occurrence of Ca\(^{2+}\) waves and a longer latency period in the presence of ET-1 stimulation. We separated these two groups based on the change of spontaneous Ca\(^{2+}\) waves activity (SCaW) after application of ET-1 (Fig. 2 C,D), namely, on the observation that the mean SCaW activity of cells in the increasing group, is only half the mean SCaW activity of the decreasing group. ET-1 affects, in a dual manner, the SCaW frequency and latency depending on the initial SCaW activity. Low SCaW activity in control conditions leads to an increase of SCaW frequency (\( p = 0.0009 \)) and a decrease of SCaW latency (\( p = 0.0545 \)) in presence of ET-1. Opposite effects are induced when cells depart from a high frequency of SCaW. ET-1 decreases SCaW (\( p = 0.0002 \)) and increases SCaW latency (\( p = 0.0024 \)). These changes were prevented by 2-APB (Supplemental fig. 3).

We have hypothesized that IP\(_3\)ICR in combination with additional regulatory factors may affect the Ca\(^{2+}\) dependent open probability of RyRs. In other words, shifting the Ca\(^{2+}\)-open probability curve of the RyRs to lower intracellular Ca\(^{2+}\) concentrations. IP3R2s and RyR2s are...
most likely co-expressed in cardiomyocytes and may form microclusters. However, RyR2s normally have a very low open probability (P_o) at resting cytosolic Ca^{2+} concentrations (i.e. at the time of the diastole). During each action potential the P_o of the RyR2 is substantially increased by Ca^{2+} entering via L-type Ca^{2+} channels. Now, long lasting Ca^{2+} release by IP3Rs in the close micro-environment of RyR2s (or dyad) which is not synchronized by AP’s, effectively increase the local [Ca^{2+}] above and beyond what it would be otherwise. This will result in a gain-of-function on the level of RyR2s. In other words, the RyRs P_o curve is left-shifted due to additional Ca^{2+} priming the local Ca^{2+} concentration. IP3Rs are not really more Ca^{2+} sensitive but they appear to be more sensitive. In addition, the timing and duration of IP3-ICR interference in regulating the Ca^{2+} sensitivity of RyRs openings appears to be of crucial importance for the modulatory effect on CICR and the global Ca^{2+} homeostasis in cardiomyocytes. In other words, the initial remodelling situation at the cellular level and time frame when IP3-ICR interacts/interferes with the CICR machinery is critical for the resulting modulatory effect of IP3 on the global (cell-wide) Ca^{2+} signaling. On the one hand, the effect can be supportive for the overall CICR global transient formation in case of an insufficiently working CICR machinery, on the other hand it could work in a protective way (anti-arrhythmogenic) by increasing the SR-Ca^{2+} leak to keep the cardiomyocytes functioning as long as possible, even at the cost of reduced CICR efficiency. However, we have already shown that in WT animals the functional impact of IP3-ICR on ECC is small, if not negligible. This situation changes under conditions of increased functional IP3R2 expression under pathophysiological conditions as seen in the used IP3R over-expression model used here. Taken together, our results suggest that increased expression of IP3R in cardiomyocytes and the corresponding increased IP3-ICR in the context of ECC remodeling under pathophysiological conditions could have a dual action in the functional modulation of CICR. Based on their individual ScAw appearance and activity/history, respectively, cardiomyocytes can respond in essentially opposing ways: pro- or anti-arrhythmogenic with the consequence of boosting or curtailing CICR chain reactions. Depending on the stage and progress of the cardiac phenotype IP3-ICR could be a regulatory cellular mechanism switching between a supportive or protective “mode”.

In conclusion, our findings support the hypothesis that IP3-Ca^{2+} release can both support and inhibit the CICR mechanism. The latter mechanism helps protecting the myocardium from arrhythmogenic episodes or assists in triggering CICR to increase myocardial contraction. The direction of this regulation is largely determined by the initial Ca^{2+} level during the progression of cardiac disease and the corresponding myocyte remodeling [7].

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use generative AI or AI-assisted technologies in the development of this manuscript.

Declaration of Competing Interest

None.