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MiR-1 Overexpression Enhances Ca^{2+} release and Promotes Cardiac Arrhythmogenesis by Targeting PP2A Regulatory Subunit B56α and Causing CaMKII-Dependent Hyperphosphorylation of RyR2

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Abstract

MicroRNAs are small endogenous noncoding RNAs that regulate protein expression by hybridization to imprecise complementary sequences of target mRNAs. Changes in abundance of muscle-specific microRNA, *miR-1*, have been implicated in cardiac disease, including arrhythmia and heart failure. However, the specific molecular targets and cellular mechanisms involved in the action of *miR-1* in the heart are only beginning to emerge. In this study we investigated the effects of increased expression of *miR-1* on excitation-contraction coupling and Ca^{2+} cycling in rat ventricular myocytes using methods of electrophysiology, Ca^{2+} imaging and quantitative immunoblotting. Adenoviral-mediated overexpression of *miR-1* in myocytes resulted in a marked increase in the amplitude of the inward Ca^{2+} current, flattening of Ca^{2+} transients voltage dependency and enhanced frequency of spontaneous Ca^{2+} sparks while reducing the sarcoplasmic reticulum Ca^{2+} content as compared with control. In the presence of isoproterenol, rhythmically paced, *miR-1*-overexpressing myocytes exhibited spontaneous arrhythmogenic oscillations of intracellular Ca^{2+}, events that occurred rarely in control myocytes under the same conditions. The effects of *miR-1* were completely reversed by the CaMKII inhibitor KN93. Although phosphorylation of phospholamban was not altered, *miR-1* overexpression increased phosphorylation of the ryanodine receptor (RyR2) at S2814 (CaMKII) but not at S2808 (PKA). Overexpression of *miR-1* was accompanied by a selective decrease in expression of the protein phosphatase PP2A regulatory subunit B56α involved in PP2A targeting to specialized subcellular domains. We conclude that *miR-1* enhances cardiac excitation-contraction coupling by selectively

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DISCLOSURES

None.

increasing phosphorylation of the L-type and RyR2 channels via disrupting localization of PP2A activity to these channels.

Keywords

Ryanodine receptor; *miR-1*; CaMKII; PP2A; arrhythmia

INTRODUCTION

Cardiac contractility relies on release of Ca^{2+} from the sarcoplasmic reticulum (SR) and alterations in intracellular Ca^{2+} cycling have been implicated in different cardiac diseases, including arrhythmia and heart failure (HF). Normally, SR Ca^{2+} release is activated by Ca^{2+} that enters the cell through voltage-dependent Ca^{2+} channels of the sarcolemma during the plateau phase of the cardiac action potential (AP). This process, known as Ca^{2+}-induced Ca^{2+} release (CICR)[1], involves the ryanodine receptor (RyR2) channels on the SR and is essential for activation of contractile filaments during myocardial contraction[2]. Relaxation occurs when Ca^{2+} released to the cytosol is re-sequestered to the SR by the phospholamban-controlled SR Ca^{2+} ATPase (SERCA). Cardiac contractility is modulated by reversible phosphorylation of the components of SR Ca^{2+} release machinery, including the L-type Ca^{2+} channel (dihydropyridine receptor, DHPR), RyR2 and phospholamban (PLB), by protein kinase A (PKA)[2, 3], Ca^{2+}/calmodulin-dependent protein kinase (CaMKII)[4, 5] and phosphatases PP1 and PP2A[6–8]. While in general both PKA and CaMKII potentiate SR Ca^{2+} release and enhance contractility[2], the underlying mechanisms of these effects, and in particular the role of RyR2 phosphorylation/dephosphorylation, remain highly controversial[3, 9–14].

MicroRNAs are recently discovered molecules consisting of ~22 nucleotides that regulate gene expression by annealing to target messenger RNAs inhibiting translation or promoting mRNA degradation[15]. Of approximately 600 microRNAs identified in vertebrates, several, including *miR-1*, are muscle-specific[16–18]. *MiR-1* has been shown to be involved in cardiac development and apoptosis[17, 18] and is reportedly upregulated in hearts from individuals with coronary artery disease[19] and HF[20]. Several targets for *miR-1* have been identified in the heart, including connexin 43 and Kir2.1 and *miR-1*-mediated reductions in expression of these proteins has been implicated in arrhythmogenesis[19]. However, considering the inherent capacity of miRNAs to target a broad range of proteins, the link between *miR-1* and HF and sudden cardiac death is far from being clear and more *miR-1* targets involved in these disease states are likely remain to be identified. In the present study we investigated the effects of increased expression of *miR-1* on excitation-contraction coupling and Ca^{2+} cycling in rat ventricular myocytes by using methods of cellular electrophysiology and Ca^{2+} imaging. Our results identified a new potentially important target for *miR-1* in the heart, namely the PP2A regulatory subunit B56α. Through translational inhibition of this mRNA target, *miR-1* causes CaMKII-dependent hyperphosphorylation of RyR2, enhances RyR2 activity, and promotes arrhythmogenic SR Ca^{2+} release.

MATERIALS AND METHODS

The cellular and subcellular effects of adenovirally mediated *miR-1* overexpression were studied in isolated adult rat ventricular myocytes maintained in culture for 36–48 hrs. Cytosolic Ca^{2+} changes were monitored using confocal microscopy, and whole cell currents and membrane potential were recorded with the patch-clamp technique. Changes in levels of RNA, protein expression and protein phosphorylation were studied using standard approaches.

An expanded Materials and Methods section can be found in the online data supplement available at http://circres.ahajournals.org.

RESULTS

MiR-1 Stimulates I_{Ca} and SR Ca^{2+} Release in Cardiac Myocytes

Myocytes were infected with either an adenoviral construct for expression of *miR-1* (Ad-*miR-1*)[21] or a construct containing a nontranslatable DNA segment that served as control (Ad-control). As determined by RT PCR, *miR-1* abundance was increased ~2 fold in myocytes infected with Ad-*miR-1* compared with control cells (Online Figure I). First, we investigated the effects of overexpression of *miR-1* on EC coupling in voltage clamped cardiac myocytes. Inward Ca^{2+} currents (I_{Ca}) and intracellular Ca^{2+} transients were simultaneously measured in cardiac myocytes depolarized to membrane potentials in the range between –40 and 60 mV (Fig. 1). Overexpression of *miR-1* resulted in a significant increase in the amplitude of I_{Ca} at membrane potentials of -40 to 20 mV (Fig. 1*B*). Additionally, the voltage-dependence of I_{Ca} (I–V curve) was shifted to the left 4 mV in *miR-1* myocytes (Online Figure II). Despite increased I_{Ca}, the trigger for SR Ca^{2+} release, maximum Ca^{2+} transient amplitude was not changed by *miR-1* overexpression (Fig. 1*A,B*). The failure of enhanced I_{Ca} to increase maximum SR Ca^{2+} release is attributable to reduced SR Ca^{2+} content limiting Ca^{2+} release in *miR-1* cells (see below). At the same time, the voltage-dependence of Ca^{2+} transients was markedly broadened and flattened compared with control due to increased Ca^{2+} transient amplitude at small and large depolarizations (Fig. 1*B*). While the increase of Ca^{2+} transient amplitude at small depolarizing pulses can be accounted for by increased I_{Ca}, the increase of Ca^{2+} transients at large depolarizing steps occurred without an increase in I_{Ca} and is indicative of enhanced responsiveness of Ca^{2+} release channels to I_{Ca} (i.e. enhanced EC coupling gain).

The effects of *miR-1* overexpression on the SR Ca^{2+} release were further studied by measuring spontaneous local Ca^{2+} release events, Ca^{2+} sparks, in intact quiescent myocytes loaded with Fluo-3 AM (Fig. 2). Spark frequency in *miR-1* overexpressing cells was significantly higher than in control cells (4.39±0.54 and 2.88±0.41 100 μm^{-1} s^{-1} respectively, Fig. 2*A,C*). We assessed the Ca^{2+} loading state of the SR in *miR-1* vs. control myocytes by application of caffeine. Judging from the amplitude of caffeine-induced Ca^{2+} transients, SR Ca^{2+} content was reduced to 75% of control in *miR-1* myocytes (Fig. 2*B,D*). The lowered SR Ca^{2+} content is a likely result of increased spark-mediated SR Ca^{2+} leak and it provides an explanation for the lack of potentiation of maximum Ca^{2+} release by increased I_{Ca} in *miR-1* overexressing myocytes. Therefore, *miR-1* caused profound changes

in EC coupling, including increased I_{Ca}, enhanced RyR2 channel functional activity and a reduction in the SR Ca^{2+} content.

The Stimulatory Effects of *MiR-1* in I_{Ca} and SR Ca^{2+} Release are Caused by Phosphorylation of DHPRs and RyR2s

The effects of *miR-1* overexpression upon I_{Ca} in cardiomyocytes, namely the increased I_{Ca} amplitude and left-ward shift of I_{Ca} voltage dependence (Fig. 1*B,C*, Online Figure II), are similar to the effects of β-adrenergic stimulation. To examine whether the action of *miR-1* involves the same mechanisms that mediate the response to β-adrenegic stimulation, we investigated the effects of the β-adrenergic agonist isoproterenol (ISO) on I_{Ca} and Ca^{2+} transients in *miR-1* vs. control myocytes. As shown in Fig. 1*B,C*, ISO increased I_{Ca} amplitude ~2-fold in control but was virtually ineffective in *miR-1* myocytes. Similarly, while ISO resulted in a ~2-fold increase in Ca^{2+} transient amplitude in control myocytes, ISO failed to cause significant changes in the amplitude and voltage-dependence of Ca^{2+} transients in *miR-1* myocytes. These results could be explained by *miR-1* and ISO acting through the same intracellular signaling mechanisms resulting in phosphorylation of target proteins including DHPR and RyR2.

RyR2 is phosphorylated at least at three different sites: at S2808 by PKA[22] and possibly CaMKII[23]; at S2814 by CaMKII[22], and at S2030 by PKA[24]. To test directly the hypothesis that *miR-1* overexpression results in increased phopshorylation of RyR2 at these sites, we quantified RyR2 phosphorylation using phosphospecific antibodies. *MiR-1* overexpression caused a marked increase in phosphorylation at the CaMKII site S2814 while leaving phosphorylation of the PKA site S2808 unaltered. The involvement of CaMKII in RyR2 phosphorylation at S2814 was further confirmed by the ability of KN93, a CaMKII inhibitor, to prevent phosphorylation at this site in *miR-1* myocytes (Fig. 3*A,B,C*). Interestingly, RyR2s were not detectibly phosphorylated at S2030 either in the control or *miR-1* groups even when the myocytes were exposed to 100 nmol/L ISO (Online Figure III). *MiR-1* did not change phopshorylation of PLB at its PKA or CaMKII sites, S16 and T17, respectively (Fig. 3*A,D*) but increased phosphorylation of DHPRs (Fig. 3*A,E*).

To test the involvement of CaMKII in the functional effects of *miR-1* on SR Ca^{2+} release in myocytes, we examined the impact of KN93 on *miR-1*-dependent changes in Ca^{2+} cycling. KN93 reversed the effects of *miR-1* on frequency of Ca^{2+} sparks and on the SR Ca^{2+} content of myocytes (Fig. 2), as well as on Ca^{2+} currents and Ca^{2+} transients in voltage-clamped cells (Online Figure IV). Collectively, these results suggest that *miR-1* overexpression results in augmented phosphorylation of DHPR and RyR2 by CaMKII, while the phosphorylation of PLB is unaltered.

MiR-1 Inhibits Expression of the PP2A Regulatory Subunit B56α

The phoshorylation-dependent effects of *miR-1* on Ca^{2+} handling point to the possibility that *miR-1* targets components of the phosphorylation-dephosphorylation system in myocytes. Bioinformatic sequence analysis revealed that the PP2A regulatory subunit B56α is a potential target of *miR-1* since it harbors 7-basepair long complimentary seed sequence in its 3′-UTR region (Fig. 4*A*). Binding of *miR-1* to B56α encoding RNA was confirmed by a

luciferase reporter assay (Fig. 4*B*). Moreover, the *miR-1* targeting of B56α in cardiac myocytes was confirmed by quantitative immunoblot analysis using an anti-B56α antibody (Fig. 5*B,C*). Importantly, *miR-1* overexpression did not appreciably change expression levels of a number of relevant Ca^{2+} and phosphorylation handling proteins, including DHPR, RyR2, SR Ca ATPase (SERCA2a), PLB, sodium/calcium exchanger (NCX1), calsequestrin (CASQ2), catalytic subunits of PP1, PP2A and CaMKII, as well as phosphodiesterases 3A and 4D. As shown in Fig. 5*A* expression of all of these proteins was similar in *miR-1* vs. control myocytes. Of note, *miR-1* overexpression resulted in a decrease in expression of PKA β catalytic subunit, which is also a predicted target of *miR-1*. However, down-regulation of this kinase cannot account for the increase in phosphorylation of target proteins by *miR-1* in our experiments.

An important condition for B56α to be able to specifically influence phosphorylation of the DHPR and RYR2 channels is that it must be in close proximity to these channels. To examine the cellular distribution of B56α with respect to RyR2 in cardiac myocytes, we performed immunostaining experiments. As demonstrated in Fig. 5*D*, B56α shows a substantial degree of co-localization with RyR2 at the Z-lines. Thus, B56α and RyR2 (and hence DHPR) appear to coexist in specific subcellular compartments of ventricular myocytes. *MiR-1* overexpression reduced B56α abundance uniformly without a change in the overall distribution pattern across the cells. In accordance with the targeting role of B56α, the distribution of PP2A catalytic subunit changed from localized preferentially at the Z-lines to more diffused following overexpression of *miR-1* (Online Figure V).

β-Adrenergic Stimulation Promotes Arrhythmia in *MiR-1* Overexpressing Myocytes

RyR2 hyperphosphorylation by either PKA or CaMKII has been suggested to present a possible mechanism for Ca^{2+}-dependent arrhythmia, including catecholaminergic polymorphic ventricular tachycardia (CPVT)[25, 26]. To determine whether *miR-1* overexpression renders myocytes prone to arrhythmogenic alterations in Ca^{2+} cycling, we examined *miR-1* and control myocytes as to the occurrence of spontaneous, extrasystolic Ca^{2+} transients and associated delayed afterdepolarizations (DADs)[27, 28] (Fig. 6, Online Figure VI). Control and *miR-1* myocytes were paced at 1 Hz in the presence or absence of ISO (100 nmol/L). In the presence of ISO, the two groups showed no significant differences in the amplitude of Ca^{2+} transients and SR Ca^{2+} load when paced at 1 Hz (Fig. 6*A,B*). Control myocytes exposed to ISO exhibited relatively few Ca^{2+} waves and DADs. However, the number of spontaneous extrasystolic Ca^{2+} waves per second increased more than 10-fold in *miR-1* overexpressing cells compared with controls (Fig. 6*C*). Simultaneously with Ca^{2+} waves, these cells exhibited DADs (Online Figure VI). Remarkably, incubation of *miR-1* overexpressing myocytes with 1 μmol/L KN93 completely abolished the *miR-1*-mediated boost in arrhythmogenic activity (Fig. 6*A,C*). In the absence of ISO, spontaneous Ca^{2+} waves were only rarely observed in paced *miR-1* myocytes. However, the properties of SR Ca^{2+} release in these cells were strikingly altered compared to control. The amplitude of Ca^{2+} transients in *miR-1* myocytes was ~2-fold higher than in control (Online Figure VII). Additionally, *miR-1* myocytes exhibited substantial spontaneous diastolic SR Ca^{2+} release, seen as Ca^{2+} sparks, much less pronounced in control cells (see insets in Online Figure VII). Collectively, these results suggest that *miR-1* overexpression renders RyR2s hyperactive

even in the absence of PKA phosphorylation and that arrhythmogenic spontaneous Ca^{2+} release is linked to RyR2 phosphorylation by CaMKII rather by PKA.

DISCUSSION

In the present study, we investigated the effects of the muscle-specific microRNA, *miR-1*, on excitation-contraction coupling and regulation of SR Ca^{2+} release in cardiac myocytes. Our major findings are that *miR-1* enhanced the functional activity of RyR2 channels and thus resulted in increased EC coupling gain, elevated diastolic SR Ca^{2+} leak, and reduced SR Ca^{2+} content, and promoted arrhythmogenic disturbances in myocyte Ca^{2+} cycling. These effects were attributable to translational inhibition by *miR-1* of the B56α regulatory subunit of protein phosphatase PP2A in turn causing hyperphosphorylation of RyR2 by CaMKII. These findings have important implications for the functional role of microRNAs and mechanisms of arrhythmogenesis in cardiac muscle.

Our conclusion that *miR-1* altered myocyte Ca^{2+} signaling through disruption of site-specific PP2A phosphatase activity was supported by the following results. 1) *miR-1* expression in myocytes resulted in reduced expression of B56α involved in subcellular localization of PP2A activity; 2) *miR-1* increased phosphorylation of the DHPR and RyR2 without affecting phosphorylation of PLB localized in a separate cellular domain; 3) B56α co-localized with RyR2 in myocytes; and 4) The functional effects of *miR-1* on EC coupling and intracellular Ca^{2+} cycling were reversed by the inhibitor of CaMKII KN93. The PP2A catalytic subunit has been shown to complex with DHPR[7] and RyR2[8] and is critical to dephosphorylation of these proteins following their phosphorylation by PKA and/or CaMKII[7, 8]. Consistent with the role of B56α in conveying PP2A phosphatase activity to DHPR, B56α has been shown to co-immunoprecipitate with the α subunit of the cardiac L-type Ca^{2+} channel ($Ca_v1.2α$)[29]. As to RyR2, it has been previously reported that the PP2A catalytic subunit associates with the RyR2 complex through the regulatory subunit, PR130, which binds directly to RyR2 by a leucine zipper motif[30]. However, since PR130 does not present a target for *miR-1*, it is unlikely to play a role in the effects of *miR-1* in our experiments. In support of the involvement of B56α, it has been shown to interact with the anchoring protein, ankyrin-B[31], which in turn binds to RyR2[32] thereby linking PP2A activity to the RyR2. Of note, myocytes from genetically altered mice lacking ankyrin-B exhibit increased propensity for spontaneous Ca^{2+} release and DADs when challenged with ISO similar to the effects of *miR-1* in our study (see for discussion below). Moreover, human mutations in ankyrin-B have been linked to CPVT an arrhythmic disorder associated with abnormal Ca^{2+} handling and DADs[33, 34]. Based on our findings, disruption of PP2A activity may contribute to, or account for, those abnormalities.

The RyR2 is phosphorylated by PKA and CaMKII at a number of different sites[22–24]. In our study the alterations of RyR2 function and SR Ca release in *miR-1* overexpressing myocytes appear to be caused by CaMKII rather than PKA for the following reasons. First, the potentiation of RyR2 function was associated with increased phosphorylation of RyR2 at the CaMKII site S2814 but not at the PKA site S2808, as determined by phosphospecific antibodies directed to these sites. Furthermore, the effects of *miR-1* could be prevented by the CaMKII inhibitor KN93. The observed changes in myocyte Ca^{2+} handling, including

increased EC coupling gain, reduced SR Ca^{2+} content and increased SR RyR2-mediated Ca^{2+} leak are similar to those observed by others in cells overexpressing CaMKII[26] and are consistent with *miR-1* effects being mediated by CaMKII. Thus, collectively, these results suggest that *miR-1* influences myocyte Ca^{2+} signaling by regulating the spatial distribution of phosphatase PP2A activity and hence promoting RyR2 phosphorylation by CaMKII.

Additionally, phosphorylation of the L-type Ca^{2+} channel (α1C and/or β subunits of DHPR) by PKA, PKC and CaMKII is known to occur at a number of sites and cause potentiation of the L-type Ca^{2+} current[4, 7, 29, 35]. At the present time, we do not know which of the specific phosphorylation pathways and sites are involved in modulation of I_{Ca} by *miR-1* in our study. In principle, by targeting PP2A activity in the vicinity of the L-type Ca^{2+} channels, *miR-1* would be expected to enhance phosphorylation at all sites. The failure of ISO to further stimulate the enlarged I_{Ca} in *miR-1* cells (Fig. 1) supports the possibility that PKA plays a role in mediating the effects of *miR-1 on* I_{Ca}. At the same time, the fact that CaMKII inhibition attenuates the *miR-1*-induced increase in I_{Ca} (Online Figure IV) points to the possibility that CaMKII might be also involved. Future studies using genetic mouse models with targeted mutations of the phosphorylation sites on the L-type Ca^{2+} channel may help to resolve this issue.

An important finding of this work is that *miR-1* resulted in profound disturbances in myocyte Ca^{2+} cycling manifested as extrasystolic spontaneous Ca^{2+} releases. Spontaneous Ca^{2+} releases are arrhythmogenic as they results in DADs and EADs[27, 28]. DADs and EADs in turn present triggering events for arrhythmia. Interestingly, these abnormal Ca^{2+} releases typically occurred after stimulation of the *miR-1* overexpressing cells with ISO and were only rarely observed in ISO-treated control myocytes or untreated *miR-1* overexpressing cells. Correlating these functional effects with the state of RyR2 phosphorylation at S2808 and S2814 revealed that disrupted Ca^{2+} cycling was associated with maximum phosphorylation at S2814 but not necessarily at S2808, and again this abnormal Ca^{2+} cycling was completely preventable by inhibition of CaMKII. The increased occurrence of global spontaneous Ca^{2+} waves and DADs in *miR-1* cells in the presence of ISO is likely to be due to stimulation of SERCA-mediated SR Ca^{2+} uptake rather than caused by additional effects upon CAMKII-phosphorylated RyR2s. The stimulatory effects of CaMKII on SR Ca^{2+} release and the involvement of CaMKII in the pro-arrhythmic effects of *miR-1* are consistent with previous studies which showed that CaMKII activation is involved in arrhythmia induction in various pathologic settings including cardiac hypertrophy and heart failure[26, 36].

Previously, we reported that exogenous phosphatases, including PP2A, elevate cardiac SR Ca^{2+} leak by stimulation of RyR2s[10]. This result is in apparent contradiction with the stimulatory effects of increased RyR2 phosphorylation described in the present study. Recently, we found that both phosphorylation and dephosphorylation can stimulate RyR2 activity resulting in increased SR Ca^{2+} leak in cardiomyocytes[37]. Although we do not have a definitive explanation for these apparently contradicting results, they can be rationalized by considering that RyR2 is a multimer with multiple sets of phosphorylation sites. It is possible that RyR2 activity is lowest at intermediate phosphorylation of a certain set of sites (i.e. S2814) whereas both hypo- or hyper-phosphorylation of the sites leads to increased

activity. It is also possible that the observed stimulatory effects of kinases and phosphatases are mediated by different sets of sites. Elucidation of the precise mechanisms of action of phosphorylation and dephosphorylation on RyR2 should await further studies.

MiR-1 levels have been shown to increase in human cardiac diseases, such as infarction and heart failure, and elevated *miR-1* levels have been implemented in the development of arrhythmia in these disease settings[19, 20]. Yang et al.[19] attributed the pro-arrhythmic effects of *miR-1* to down regulation of connexin 43 and the inward rectifier K channel (Kir2.1), which they identified as targets for *miR-1*. Our study demonstrated that altered Ca^{2+} signaling presents another potential arrhythmogenic mechanism for *miR-1*. *In vivo*, electrical remodeling and alterations of Ca^{2+} signaling are likely to act together to induce arrhythmia. For example, the probability of DADs reaching the threshold for AP potential for any given magnitude of spontaneous Ca^{2+} release would be expected to increase on inhibition of Kir2.1 due to increased membrane resistance[38].

A potential limitation of our study is that the experiments were performed using primary cultures of adult rat ventricular myocytes, which are known to change their characteristics over time in culture. We did not find a significant difference in I_{Ca} density either with or without ISO between cells cultured for 48 hrs and freshly isolated myocytes (Online Fig VIII). Therefore, the EC coupling machinery of rat cardiomyocytes during the first 48 hrs seems to be largely preserved. This result is consistent with the recent study by Banyasz et al[39], which showed that the most profound changes in morphometric and functional parameters, including T-tubular density and I_{Ca}, in rat ventricular myocytes take place after three days in culture.

In conclusion, our results identified a new important target for *miR-1* in the heart, namely the PP2A regulatory subunit B56α. Through translational inhibition of this mRNA target, *miR-1* causes CaMKII-dependent hyperphosphorylation of RyR2, enhances RyR2 activity, and promotes arrhythmogenic SR Ca^{2+} release. This mechanism could contribute to induction of arrhythmia in disease states accompanied by elevated *miR-1*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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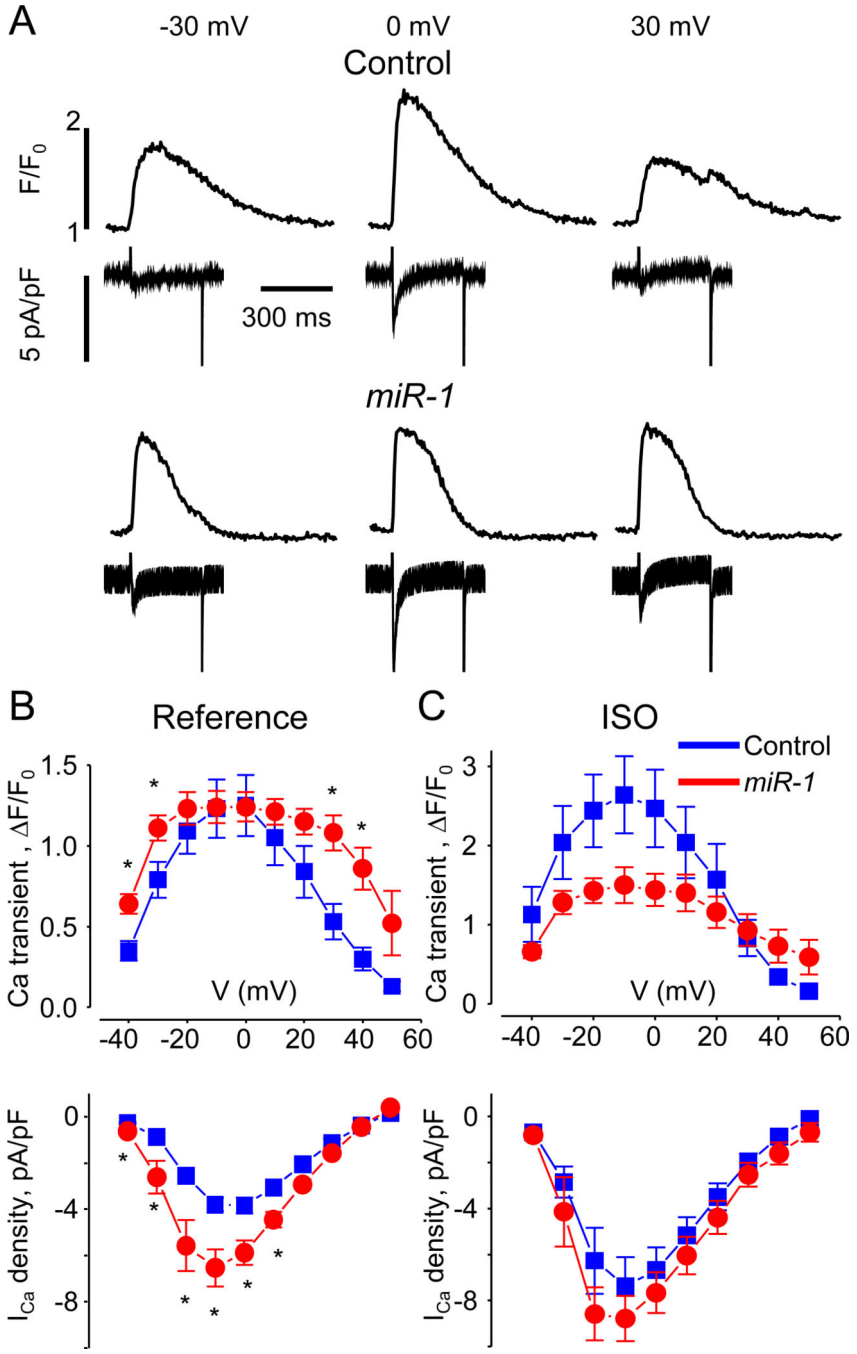

Fig. 1. *MiR-1* Overexpression Stimulates I_{Ca} and SR Ca^{2+} Release in Cardiac Myocytes

(*A*) Representative recordings of Ca^{2+} transients and I_{Ca} evoked by depolarizing steps from a holding potential of −50 mV to −30, 0 and +30 mV in a control myocyte and a myocyte overexpressing *miR-1*. The voltage steps were applied at intervals of 5 s. (*B, C*) Voltage dependencies of I_{Ca} (bottom) and Ca^{2+} transient amplitude (top) in control (blue) and *miR-1*-overexpressing (red) myocytes at baseline conditions (*B*) and upon application of 100 nmol/L ISO (*C*). *Significantly different vs. control at P<0.05, One Way ANOVA (N=5–7).

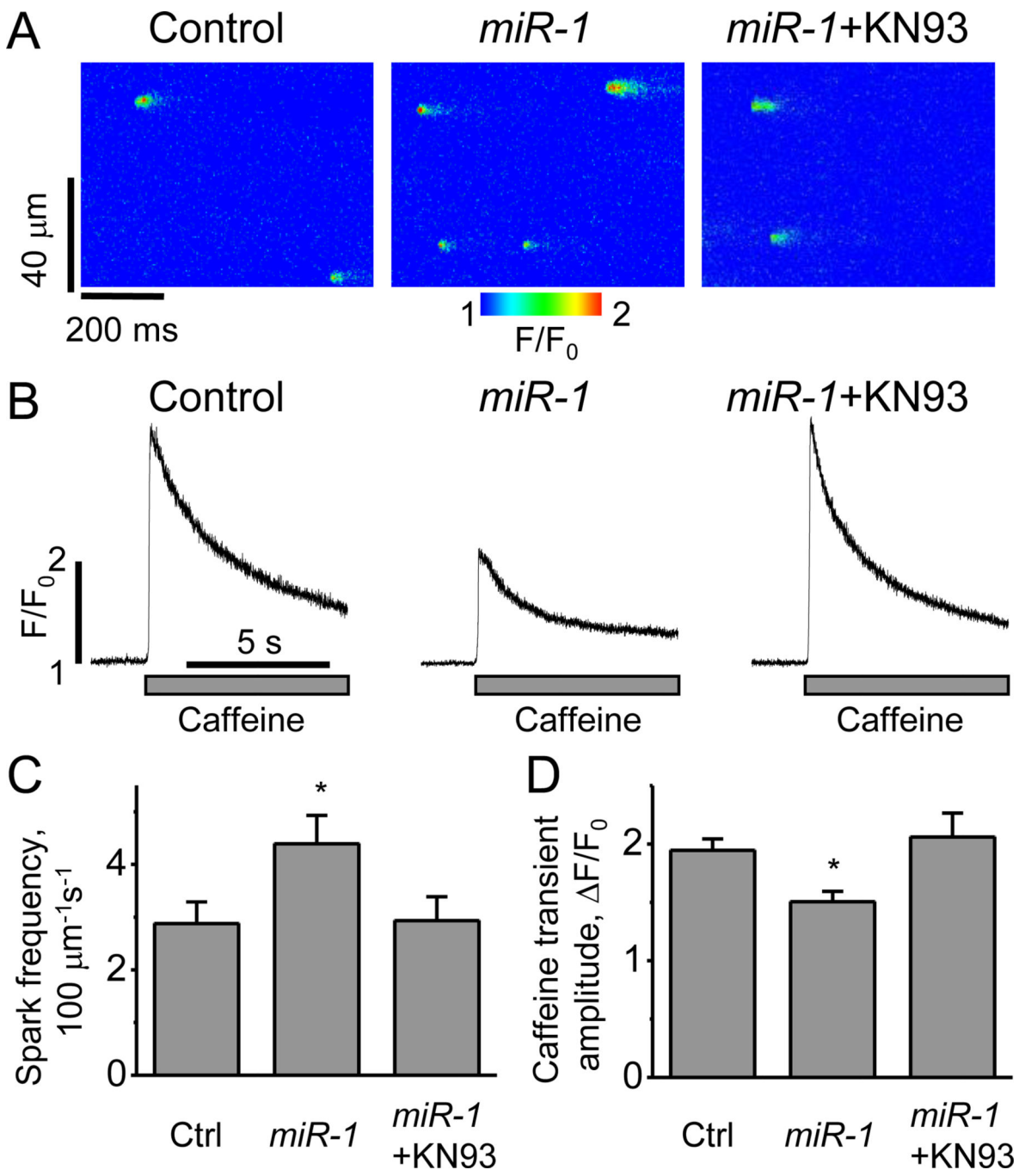

Fig. 2. *MiR-1* Overexpression Increases Ca^{2+} Spark Frequency and Decreases SR Ca^{2+} Content in Intact Cardiac Myocytes

(*A*, *B*) Representative line-scan images of Ca^{2+} sparks (*A*) and time-dependent profiles of global Ca^{2+} releases induced by application of 10 mmol/L caffeine (*B*) recorded in a control myocyte and a myocyte overexpressing *miR-1* at reference conditions and after treatment with the CaMKII inhibitor KN93 (1 μmol/L). (*C*) Averaged spark frequency for the three myocyte groups; n was 33, 53 and 45 for control, *miR-1* and *miR-1* treated with KN93, respectively. (*D*) Averaged amplitude of caffeine-induced Ca^{2+} transients; n was 14, 14 and

8 for control, *miR-1* and *miR-1* treated with KN93, respectively. *Significantly different vs. control at P<0.05, One Way ANOVA.

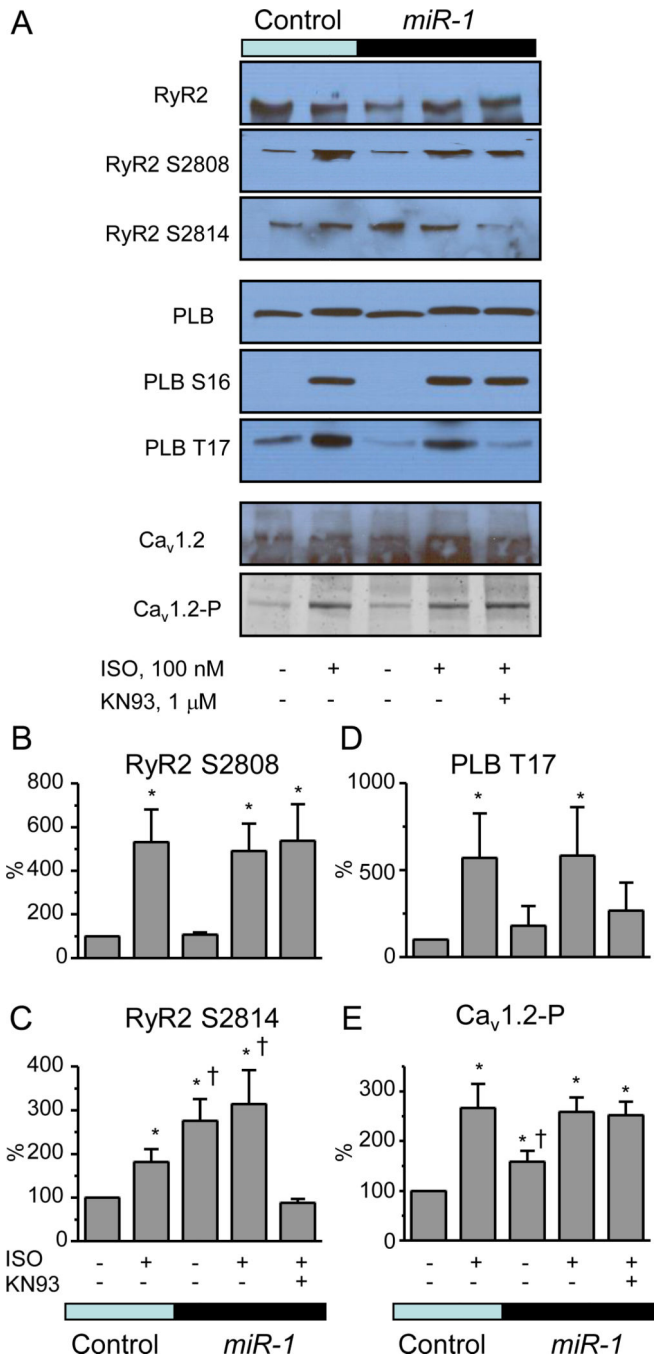

Fig. 3. *MiR-1* Overexpression Increases RyR2 and DHPR Phosphorylation and Does not Affect Phosphorylation of PLB

(*A*) Representative Western blots. RyR2 phosphorylation at sites S2814 and S2808 and PLB phosphorylation at S16 and T17 was measured with phospho-specific antibodies. DHPR phosphorylation level was assessed using the Pro-Q Diamond phosphoprotein gel stain technology (Invitrogen). Total protein content (i.e. RyR2, PLB, or DHPR) was measured in the same samples on a different blot and used as a control for loading. Ventricular cells isolated from one heart were split into 5 groups, infected with the control adenovirus or the

adenovirus encoding *miR-1* and kept in culture for 48 hrs. Subsequently, myocytes were exposed to 100 nmol/L ISO (3 min) and 1 μmol/L KN93 (30 min), paced at 1 Hz for 1 min and flash frozen. (*B*) Data pooled from 6 experiments for S2808. (*C*) Data pooled from 7 experiments for S2814. (*D*) Data pooled from 7 independent experiments for T17. (*E*) Data pooled from 5 independent experiments for phospho-DHPR. *,†Significantly different vs. control (*) or vs. control+ISO (†)at $P<0.05$, paired Student's *t* test.

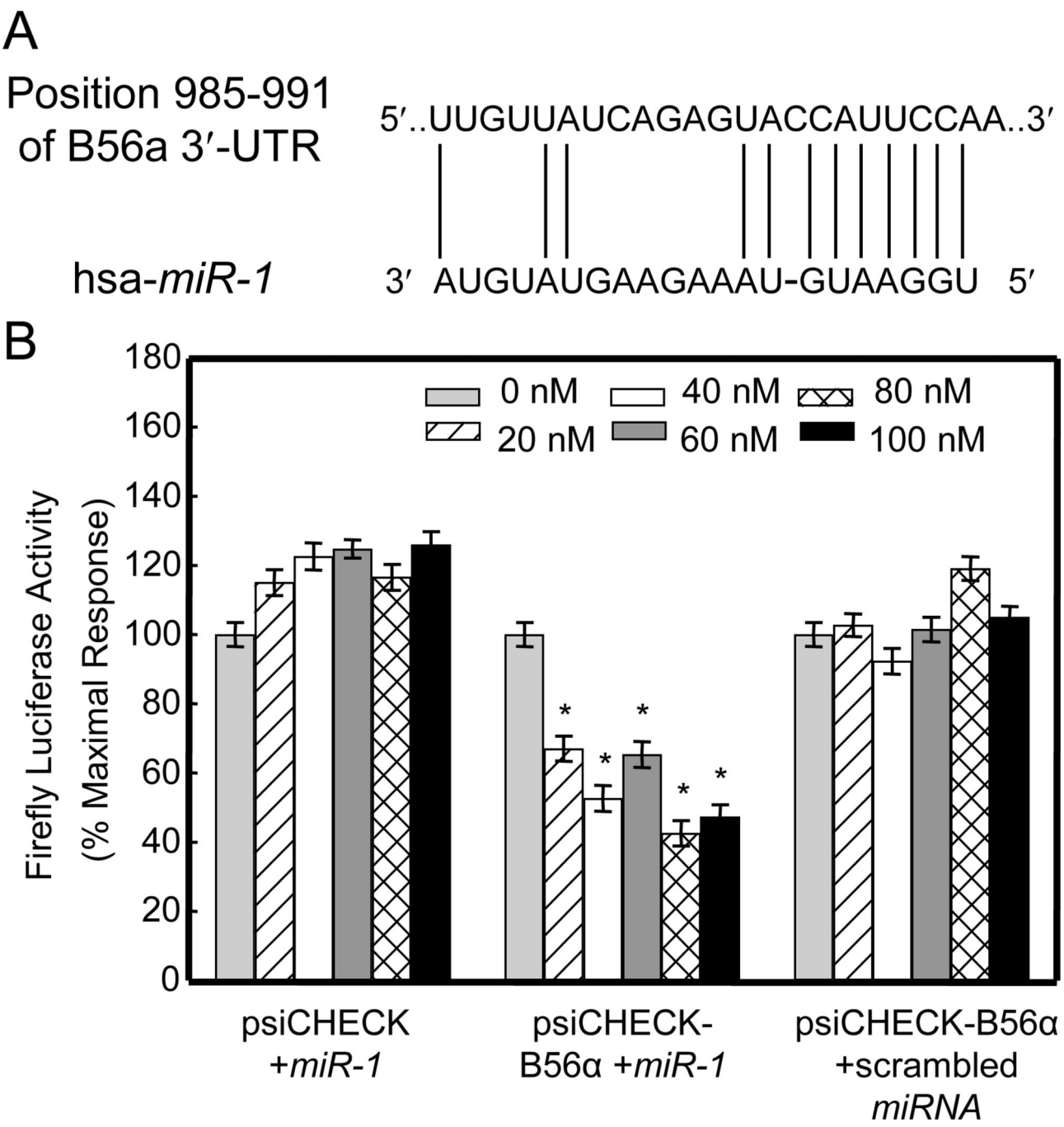

Fig. 4. PP2A Regulatory Subunit B56α is a Target for Silencing by *MiR-1*

(*A*) Complementarity between *miR-1* and the putative B56α 3′-UTR site targeted (985–991 bp downstream from the human B56α stop codon, www.Targetscan.org). The putative *miR-1* binding site harbored in the B56α 3′-UTR is conserved across species (i.e. human, mouse, rat, dog and chicken). The binding of *miR-1* to the B56α 3′-UTR target site fulfills the requirement of a 7-bp seed sequence of complementarity at the miRNA end. (*B*) CHO cells were transfected with psiCHECK or the psiCHECK-B56α luciferase reporter construct and either *miR-1* or scrambled miRNA at the concentrations indicated. Twenty-four hours

following transfection, luciferase activities were measured. Renilla luciferase activity was normalized to firefly luciferase activity and mean activities ± S.E. from five independent experiments are shown ($P<0.01$ vs. CHO cells transfected with psiCHECK-B56α at each concentration shown).

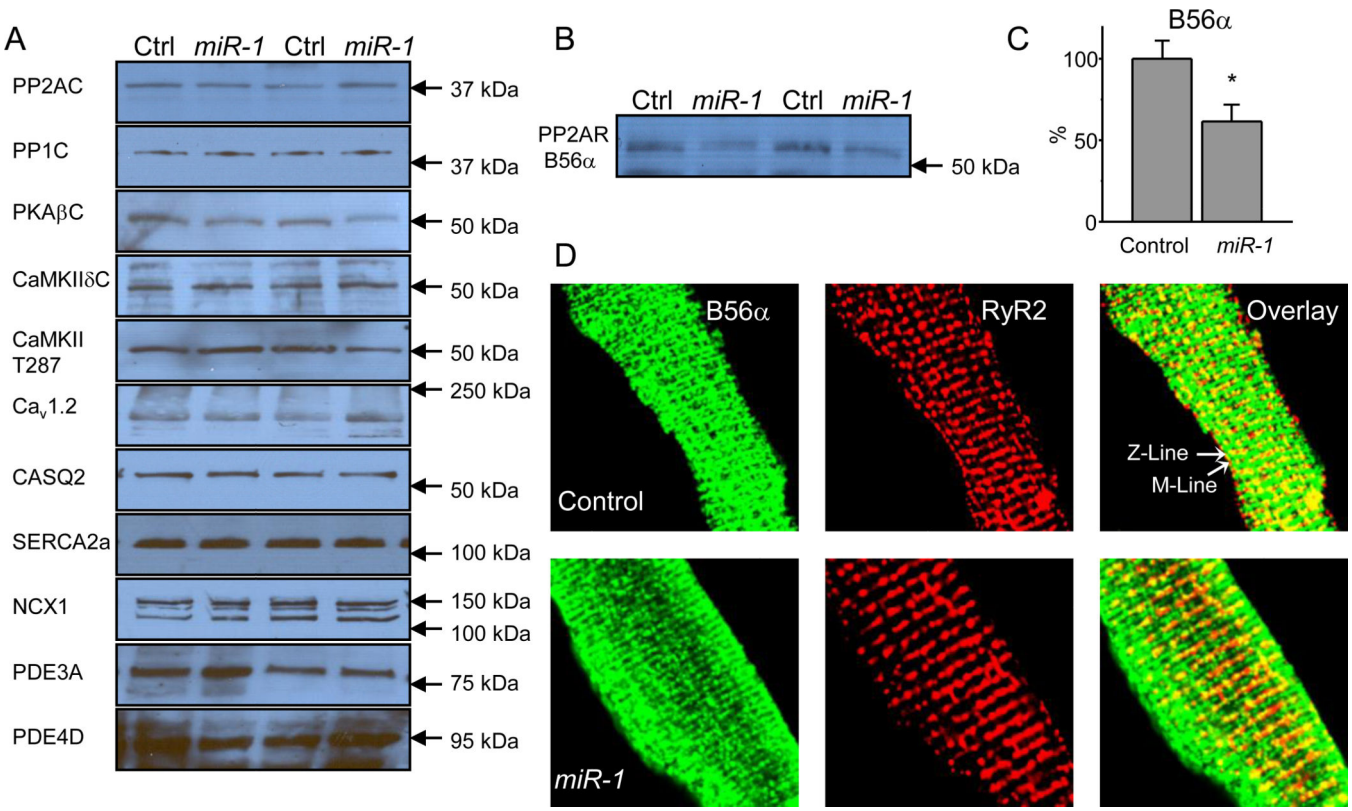

Fig. 5. *MiR-1* Overexpression Decreases Amount of PP2A Regulatory Subunit B56α in Myocytes

(*A*) Representative immunoblots of lysates from ventricular myocytes infected with control adenoviruses or adenoviruses encoding *miR-1* prepared from 2 hearts. PP2AC indicates protein phosphatase 2A catalytic subunit; PP1C, protein phosphatase 1 catalytic subunit; PKAβC, protein kinase A catalytic subunit β isoform; CaMKIIδC, Ca^{2+}-calmodulin dependent protein kinase catalytic subunit δ isoform; CaMKII T287, CaMKII phosphorylated at threonine 287 probed with phospho-specific antibody; PDE3A and PDE4D, phosphodiesterases types 3A and 4D respectively; $Ca_V1.2$, α1C subunit of L-type Ca^{2+} channel; CASQ2, cardiac isoform of calsequestrin; SERCA2a, cardiac isoform of SR Ca^{2+}-ATPase; NCX1, Na^+/Ca^{2+} exchanger type 1. 40 μg of protein per lane was used for analysis of all proteins. There is no significant difference in the density of these proteins between the 2 groups except PKAβC. (*B, C*) Representative immunoblot (*B*) and pooled data for PP2A regulatory subunit B56α in control myocytes and myocytes overexpressing *miR-1* (*C*). Data were obtained from 10 independent experiments. *Significantly different vs. control, $P<0.05$, paired Student's *t* test. (*D*) Control (top) and *miR-1*-overexpressing (bottom) cardiomyocytes fixed after 48 hours in culture coimmunolabeled with B56α- (left, green) and RyR2-specific antibodies (middle, red).

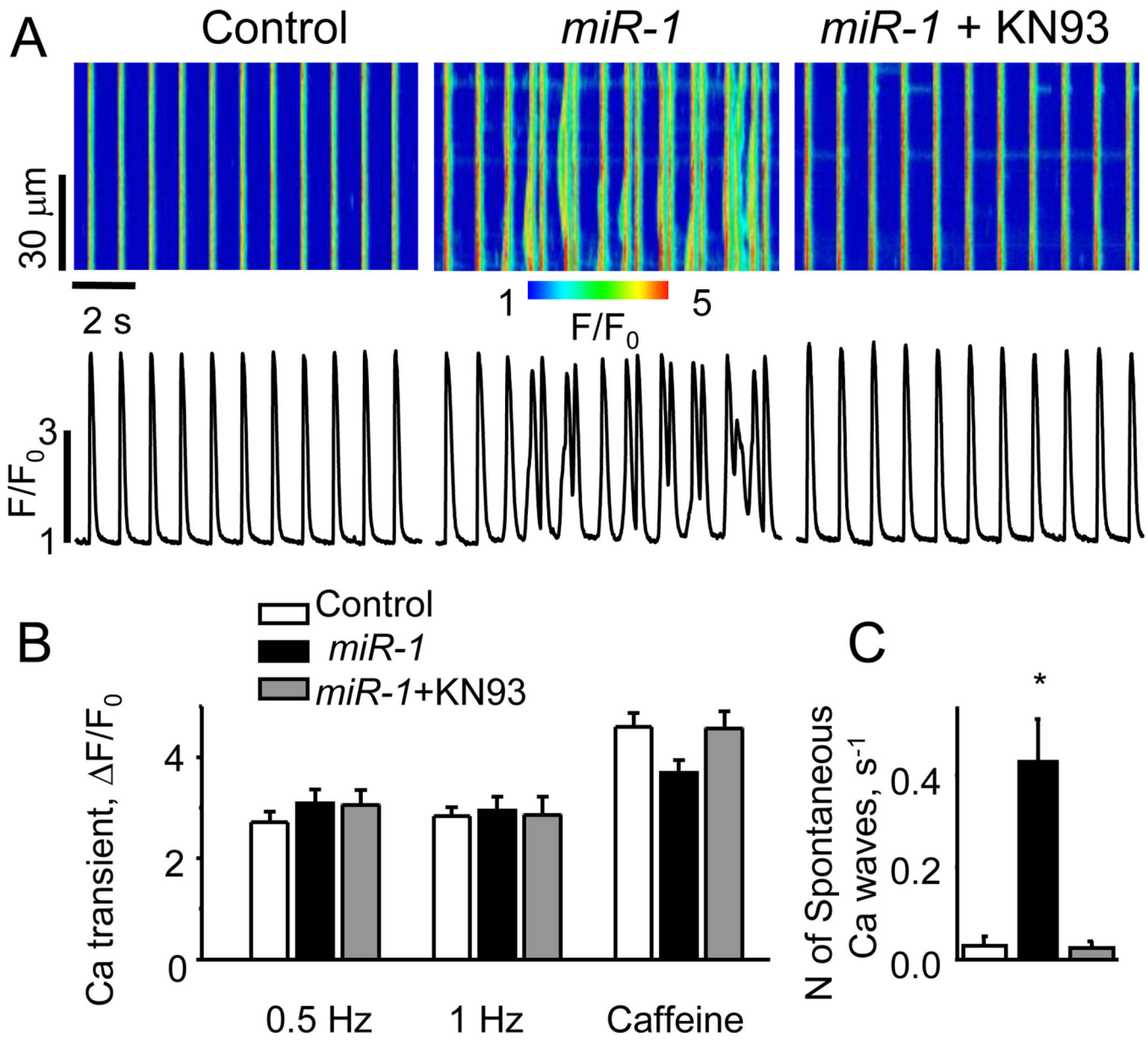

Fig. 6. *MiR-1* Overexpression Increases Arrhythmogenic Potential of Myocytes Undergoing Repetitive Stimulation in the Presence of Isoproterenol

(*A*) Representative line-scan images and corresponding time-dependent profiles of Fluo-3 fluorescence in rat ventricular myocytes infected with Ad-*Control* and Ad-*miR-1* adenoviruses. Ca transients were evoked by electrical field stimulation at 1 Hz in the presence of 100 nmol/L ISO. *MiR-1*-overexpressing myocytes displayed increased incidence of arrhythmogenic spontaneous diastolic Ca^{2+} waves. Incubation of *miR-1*-overexpressing myocytes with KN93 (1 μmol/L for 30 min) restored normal rhythmic activity. (*B*) Summarized data for Ca^{2+} transient amplitude and SR Ca^{2+} content for control, *miR-1*-overexpressing myocytes and *miR-1*-myocytes treated with KN93 in the presence of 100 nmol/L ISO. The myocytes were field-stimulated at 0.5 and 1 Hz for 1 min each then exposed 10 mmo/L caffeine. (*C*) Averaged number of spontaneous Ca^{2+} waves per second

in field-stimulated myocytes at 1 Hz in the presence of 100 nmol/L ISO. *Significantly different at $P<0.05$, One Way ANOVA. Number of cells studied (n=8–16).