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ROCK2 promotes ryanodine receptor phosphorylation and arrhythmic calcium release in diabetic cardiomyocytes☆

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ABSTRACT

Background: Diabetes is associated with an increased risk of heart failure, cardiac arrhythmias and sudden cardiac death. We previously showed that ROCK2 expression is elevated in diabetic rat hearts, and that ROCK inhibition acutely improves their contractile function. In the present study we investigated whether inhibition of ROCK or partial deletion of ROCK2 improves impaired Ca^{2+} handling in the diabetic heart.
Methods: Contractile properties and Ca^{2+} transients were measured before and after treatment with the ROCK inhibitor Y-27632 (1 μM) in fluo-4-loaded cardiomyocytes isolated from streptozotocin (STZ)-diabetic or non-diabetic rats. Cardiac function was determined *in vivo*, and contractile properties and Ca^{2+} transients also measured in cardiomyocytes from non-diabetic and STZ-diabetic wild-type (WT) and ROCK2+/− mice.
Results: ROCK inhibition improved some parameters of contractile function and Ca^{2+} handling in cardiomyocytes from diabetic rat hearts. In addition, ROCK inhibition attenuated the diabetes-induced delayed aftercontractions (DACs) and associated irregular Ca^{2+} transients induced by increased $[Ca^{2+}]o$. Although no overt cardiac dysfunction was detected in diabetic WT mice, cardiomyocytes from these mice also developed arrhythmic Ca^{2+} transients in response to increased $[Ca^{2+}]$. These were attenuated in cardiomyocytes from diabetic ROCK2+/− mice, in association with decreased diastolic Ca^{2+} leak and with reduction of the diabetes-induced increased phosphorylation of both CaMKII and the ryanodine receptor (RyR).
Conclusions: These data suggest that ROCK2 contributes to diabetes-induced impaired cardiac Ca^{2+} homeostasis, at least in part by promoting CaMKII-mediated phosphorylation of RyR. This may have important clinical implications for the treatment of the increased incidence of dysrhythmias in diabetes.

1. Introduction

Diabetic cardiomyopathy, defined as ventricular dysfunction that occurs independently of hypertension or coronary artery disease, contributes to the increased frequency and severity of heart failure in patients with diabetes [1,2]. In addition, diabetes is associated with an increased incidence of cardiac arrhythmias and with sudden cardiac death [3–5]. Impaired contractile function and arrhythmogenesis have been demonstrated *in vivo* and *ex vivo* in the isolated whole heart [6] and in isolated cardiomyocytes [7,8], indicating that they are likely due to changes occurring at the cellular and molecular level.

The RhoA/ROCK pathway has been implicated in many cardiovascular diseases, including diabetic cardiomyopathy [9]. We showed increased activation of RhoA and ROCK, associated with over-expression of the ROCK2 isoform [10] in diabetic hearts, and that acute administration of ROCK inhibitors improved ventricular function both *ex vivo* in the isolated working heart and *in vivo* in diabetic animals [9]. This occurred in the absence of an effect on heart rate or coronary flow in either control or diabetic hearts, or ventricular function in control hearts [9].

The mechanism by which acute ROCK inhibition rapidly improves contractile function is unclear. However, one possibility is that inhibition of ROCK modifies Ca^{2+} homeostasis. Impaired Ca^{2+} handling has

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been strongly implicated in diabetic cardiomyopathy [8,11,12] in which ventricular dysfunction has been associated with reduced and prolonged Ca^{2+} transients [12–14]. Impaired Ca^{2+} handling leading to intracellular Ca^{2+} overload is also associated with an increased incidence of arrhythmias in diabetes [13,15], but whether ROCK contributes to this process is unknown. This was investigated in the present study, by determining the effects of acute inhibition of ROCK and of partial deletion of ROCK2 (ROCK2+/−) on Ca^{2+} transients and contraction in cardiomyocytes isolated from diabetic and control hearts.

2. Methods

A detailed description of the methods can be found in the supplement.

2.1. Animals

Male Wistar rats (170–200 g) were treated with either with a single IV injection of 60 mg/kg STZ or citrate buffer vehicle as described in Refs. [9,10].

ROCK2+/− mice were generated as described in Ref. [16]. Complete ROCK2 knockout is highly embryonically lethal due to thrombus formation and placental dysfunction [16] and only ROCK2+/− mice were obtained. At 8 weeks of age, male ROCK2+/− mice and their WT littermate controls were treated with either STZ (40 mg kg^{-1}, intraperitoneal per day for 5 days) or an equal volume of citrate buffer vehicle.

This investigation conforms to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). All protocols were approved by the UBC Animal Care Committee.

2.2. Echocardiography

At 13 weeks post STZ treatment, mice underwent echocardiography using the Vevo 2100 system [17].

2.3. Isolation of adult rat and mouse ventricular cardiomyocytes

Ca^{2+}-tolerant adult ventricular cardiomyocytes were isolated from rat hearts as detailed in Ref. [9] and from mouse hearts using the protocol of Li et al. [18].

2.4. Measuring cell shortening/relengthening and Ca^{2+} transients in rat cardiomyocytes

Mechanical properties and Ca^{2+} transients were measured simultaneously in fluo-4 AM loaded control and diabetic cardiomyocytes superfused with buffer at 25 °C. Cells were field-stimulated at a voltage 1.5 fold above threshold (15–20 V) and a frequency of 0.5 or 1 Hz, with a stimulus duration of 2 milliseconds.

2.5. Confocal microscopy and calcium imaging of mouse cardiomyocytes

Calcium was imaged using a confocal microscope (Leica TCS SP5AOBS) as described in Ref. [19].

2.6. Measurement of diastolic Ca^{2+} leak

Sarcoplasmic reticulum (SR) Ca^{2+} leak was determined using the tetracaine method as described in Ref. [20].

2.7. ROCK activity assay

ROCK activity was measured in the absence of (control) or following 25 min treatment with 1 μM Y-27632 by determining the extent of Thr696 phosphorylation of MYPT1 in an *in vitro* assay as described in Ref. [21]. This concentration of Y-27632 is selective for ROCK over other kinases [22] and is the concentration that we previously found improved the contractile function of isolated working hearts from diabetic rats [9].

3. Results

3.1. ROCK inhibition partially restores impaired contractions and Ca^{2+} transients in cardiomyocytes from diabetic rats

We first confirmed that ROCK activity was elevated in diabetic cardiomyocytes compared to control cells and that treatment with 1 μM Y-27632 for 25 min effectively inhibited ROCK (Suppl. Fig. 1A). We next investigated the effects of Y-27632 on contraction and Ca^{2+} transients in cardiomyocytes isolated from control and diabetic rat hearts. In cells perfused with Tyrode's solution containing 2 mM Ca^{2+}, there were few significant differences in either contractile parameters or Ca^{2+} transients between cardiomyocytes from control and diabetic rat hearts (Suppl. Fig. 1B). Apart from a small decrease in peak tension and an increase in time to peak tension, no other changes in contraction were significantly different between control and diabetic cardiomyocytes. Similarly, aside from a small decrease in peak Ca^{2+}, no other parameters of the Ca^{2+} transient were significantly altered in cardiomyocytes from diabetic rats. Treatment with Y-27632 had no effect on peak tension, but significantly reduced the time to peak tension and also produced a small increase in the peak amplitude of the Ca^{2+} transient in diabetic myocytes, without affecting these parameters in control cells.

When cells were challenged by perfusion with 6 mM extracellular [Ca^{2+}], the changes in contractile function and Ca^{2+} transients in cardiomyocytes from diabetic compared to control hearts were more apparent (Fig. 1). As was found in 2 mM Ca^{2+}, the decrease in peak shortening and increase in time to peak shortening in diabetic cardiomyocytes were associated with a decrease in the peak cytosolic Ca^{2+} (Fig. 1A–C). In addition, there was a significant decrease in the rate of relaxation (Fig. 1D) and increase in the time to 50% relaxation (Fig. 1E), associated with a corresponding decrease in the rate of fluorescence decay of the Ca^{2+} transient (Fig. 1F) and increase in the time to 50% decay of the Ca^{2+} transient (Fig. 1G). Treatment of diabetic cardiomyocytes with Y-27632 again had no significant effect on peak shortening (Fig. 1A), but normalized time to peak shortening (Fig. 1B) and produced a small but significant increase in peak cytosolic Ca^{2+} (Fig. 1C) while having no effect on these parameters in control cardiomyocytes. At the same time, Y-27632 significantly improved the rate of relaxation and normalized the time to 50% relaxation. It also improved the rate of decay and the time to 50% decay of the Ca^{2+} transient in diabetic cardiomyocytes, while having no effect on relaxation in cardiomyocytes from control hearts (Fig. 1D–G). Additionally, Ca^{2+} transient propagation from the sarcolemma to the cell centre was delayed in diabetic cardiomyocytes and this was markedly improved by Y-27632 (Fig. 1H).

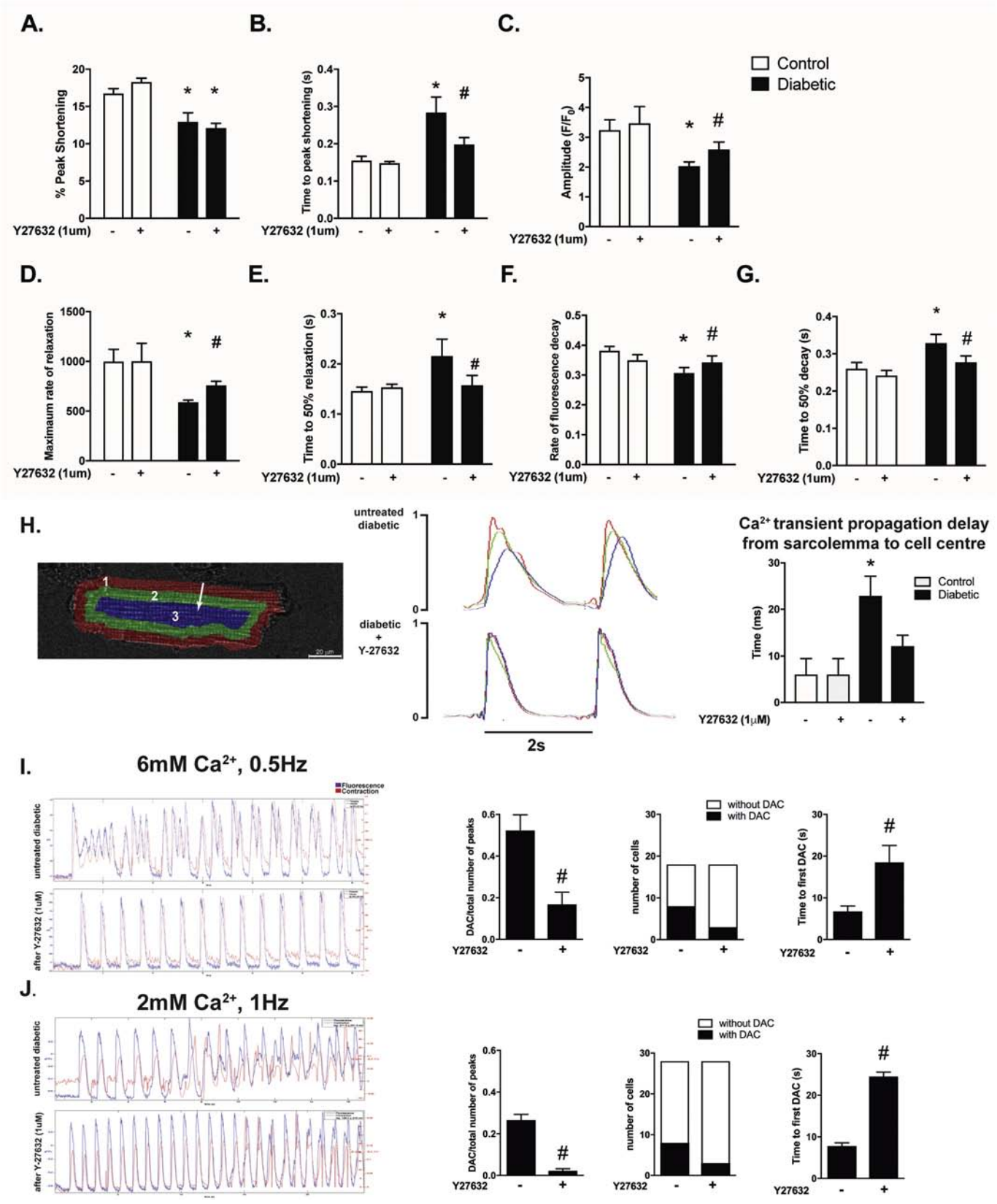
3.2. ROCK inhibition prevents diabetes-induced DACs and associated irregular Ca^{2+} transients in rat cardiomyocytes

When superfused with 2 mM Ca^{2+} and stimulated at 0.5 Hz, neither diabetic nor control cardiomyocytes developed irregular contractions. However, in the presence of 6 mM Ca^{2+}, >40% of the cardiomyocytes from diabetic hearts, but no myocytes from control hearts, displayed DACs, associated with irregular Ca^{2+} transients (Fig. 1I). Treatment with Y-27632 markedly attenuated the development of DACs, reducing the number of cells that displayed any aftercontractions by >50%

Fig. 1. Effect of Y27632 on contraction and Ca^{2+} transients in cardiomyocytes isolated from control and diabetic rat hearts. (A–G) Cardiomyocytes from control or diabetic rats were incubated for 25 min in 6 mM Ca^{2+} with or without Y27632 (1 μM) to determine the effect of ROCK inhibition on contraction and Ca^{2+}transient properties. Cells were stimulated at 0.5 Hz and only regular contractions and transients were included in the analysis. (H) Cells incubated at 6 mM Ca^{2+} were divided into 3 concentric rings (left), with the white arrow indicating the direction of transient propagation. Ca^{2+} transient propagation delay between the sarcolemma (start of the red ring) and cell centre (blue ring) was quantified at 0.5 Hz (right). The middle panel shows representative transients for the 3 rings in untreated and Y-27632 treated diabetic cardiomyocytes. (I, J) Cardiomyocytes from diabetic rats were incubated in 6 mM Ca^{2+} and stimulated at 0.5 Hz (I) or incubated in 2 mM Ca^{2+} and stimulated at 1 Hz (J) in the absence or presence of Y27632 (1 μM) to study any DACs occurring under these conditions. *$p < 0.05$ compared to control untreated. #$p < 0.05$ compared to untreated diabetic group; $n = 6–7$ rats.

(Fig. 1I). In the remaining cells, inhibition of ROCK significantly increased the time to development of the first aftercontraction from 6.75 s to 18.5 s (Fig. 1I).

Similarly, when cells were maintained in 2 mM Ca^{2+} and challenged by increasing the stimulation frequency from 0.5 to 1 Hz, diabetic but not control cardiomyocytes were also more likely to develop DACs



(Fig. 1J). Inhibition of ROCK under these circumstances almost completely abolished the development of DACs in diabetic cardiomyocytes; in the remaining cells, it prolonged the time to the first aftercontraction from 7.75 s to 24.50 s (Fig. 1J).

3.3. Partial deletion of ROCK2 prevents diabetes-induced irregular Ca^{2+} transients in mouse cardiomyocytes

We previously demonstrated that the increase in ROCK activity in hearts and cardiomyocytes isolated from diabetic rat hearts was associated with increased expression of ROCK2 [10]. Therefore, we were interested in determining whether the salutary effects of ROCK inhibition on Ca^{2+} transients in diabetic rat cardiomyocytes were also seen in myocytes isolated from ROCK2+/− diabetic mice.

The characteristics of control and diabetic WT and ROCK2+/− mice are shown in Suppl. Fig. 2A. Twelve weeks after induction of diabetes with STZ, body weights of WT and ROCK2+/− mice were similar, and significantly less than their corresponding controls. Blood glucose was markedly elevated in diabetic WT and ROCK2+/− mice, with no difference between them. As was found in hearts from diabetic rats [10], the expression of ROCK2 but not ROCK1 was significantly elevated in hearts from diabetic WT mice. On the other hand, the expression of ROCK2 was significantly less in hearts from non-diabetic ROCK2+/− than WT mice, and was not affected by the induction of diabetes. There was no change in expression of ROCK1 in hearts of ROCK2+/− mice, indicating that there was no compensatory upregulation of this isoform.

Interestingly, when measured by conventional echocardiography, no significant differences in either diastolic (E/A ratio or MV deceleration time) or systolic (fractional shortening or ejection fraction) function were detected between control and diabetic hearts from WT mice at this time (Suppl. Fig. 2B). However, using speckle-tracking strain-based echocardiography, which is a more sensitive measure of left ventricular function, we detected abnormalities in both radial and longitudinal strain rate and velocity, indicative of the onset of left ventricular wall motion abnormalities in hearts from diabetic WT mice (Suppl. Fig. 2C). These changes were not detected in hearts from diabetic ROCK2+/− mice.

No significant differences in the Ca^{2+} transient were found in cardiomyocytes isolated from diabetic WT mouse hearts compared to their non-diabetic WT controls when perfused with 2 mM Ca^{2+} (data not shown). Interestingly, when challenged with 6 mM Ca^{2+}, the peak amplitude of the Ca^{2+} transient was significantly greater in cardiomyocytes from non-diabetic ROCK2+/− compared to WT mice (Fig. 2A), although the full duration at half maximal amplitude (FDHM) was similar (Fig. 2B), as was cell shortening (Fig. 2C). Induction of diabetes had no significant effect on either the amplitude of the Ca^{2+} transient (Fig. 2A) or on cell shortening (Fig. 2C) in cardiomyocytes from ROCK2+/− mice. However, FDHM was shorter in cardiomyocytes from diabetic ROCK2+/− than WT mice (Fig. 2B). Only a small number of cells from diabetic mice displayed a delay in Ca^{2+} transient propagation to the cell centre in 6 mM Ca^{2+}. There were 5 transient delay events detected in 14 cells from 3 diabetic WT mice, compared to only 1 delay event in 21 cells from 3 diabetic ROCK2+/− mice at stimulation frequency of 1 Hz.

At the same time, as was found in cardiomyocytes from diabetic rat hearts, cardiomyocytes from diabetic WT mouse hearts were highly susceptible to the development of irregular Ca^{2+} transients when perfused with 6 mM Ca^{2+} (Fig. 3A). In diabetic WT cardiomyocytes only a few irregular transients were detected in 2 mM Ca^{2+}. However, up to 89% of diabetic WT cardiomyocytes developed arrhythmic Ca^{2+} transients when perfused with 6 mM Ca^{2+} and paced at increasing frequencies (Fig. 3A), leading to a significant prolongation of the FD at 75% maximum (Fig. 3B). These arrhythmic Ca^{2+} transients were almost completely abrogated in cardiomyocytes isolated from diabetic ROCK2+/− mice (Fig. 3A), resulting in normalization of the FD at 75% maximum (Fig. 3B).

3.4. Partial deletion of ROCK2+/− reduces Ca^{2+} leak in diabetic hearts

Since DACs and associated arrhythmias have been attributed to increased Ca^{2+} leak from the RyR2 (reviewed in Ref. [23]), we next investigated whether there was evidence of an increased Ca^{2+} leak in cardiomyocytes from diabetic WT hearts, and whether this was altered in cardiomyocytes from diabetic ROCK2+/− mice.

We first measured changes in spontaneous Ca^{2+} release in unstimulated cardiomyocytes from diabetic hearts (Fig. 3C). When cells were perfused with 2 mM Ca^{2+}, there was only limited spontaneous Ca^{2+} release in cardiomyocytes from diabetic WT hearts. However, spontaneous Ca^{2+} release events became much more prominent in diabetic WT cells perfused with 6 mM Ca^{2+}. On the other hand, there was no sign of spontaneous Ca^{2+} release in unstimulated cardiomyocytes from diabetic ROCK2+/− mice in either 2 or 6 mM Ca^{2+} (Fig. 3C).

The magnitude of the diastolic Ca^{2+} leak was next determined by measuring the tetracaine-sensitive shift in intracellular Ca^{2+} [20]. A significant increase in the Ca^{2+} leak was found in cardiomyocytes from diabetic compared to control WT mice (Fig. 4A). This increase was prevented in cardiomyocytes from ROCK2+/− mice (Fig. 4A).

3.5. The diabetes-induced increase in phosphorylation of the ryanodine receptor is prevented in ROCK2+/− hearts

The diabetes-induced increase in diastolic Ca^{2+} leak has been suggested to be due to increased phosphorylation of the RyR2 (reviewed in Ref. [23]). Therefore, we examined phosphorylation of the RyR2 in hearts from diabetic WT and ROCK2+/− mice (Fig. 4B). Although no change in total expression of the RyR2 was detected in hearts from diabetic compared to control WT mice, there was a significant increase in phosphorylation of the RyR2 at Ser 2814, the CaMKII site. However, this was completely prevented in hearts from diabetic ROCK2+/− mice. To determine whether the phosphorylation of other CaMKII targets was increased, we also examined the phosphorylation of phospholamban (PLB) at the Thr 17 site. However, no change in phosphorylation of PLB at this site was detected in hearts from either WT or ROCK2+/− diabetic mice. Lastly, we measured phosphorylation of CaMKII at Thr287, which is associated with its autonomous activation. As was found with phosphorylation of the RyR2, the phosphorylation of CaMKII was significantly increased in hearts from diabetic WT mice, but this increase was prevented in hearts from diabetic ROCK2+/− mice (Fig. 4C).

4. Discussion

We have previously shown that the activity of the RhoA/ROCK pathway and the expression of ROCK2 are elevated in hearts from diabetic rats and that acute treatment with ROCK inhibitors significantly improves cardiac contractile function [9]. In the present study, we investigated the effects of inhibition of ROCK or partial deletion of ROCK2 on Ca^{2+} transients and contractile function of cardiomyocytes isolated from diabetic hearts. The major new findings of this investigation are that: 1) in cardiomyocytes isolated from diabetic rat hearts, inhibition of ROCK partially improved impaired contractile function and Ca^{2+} transients; 2) ROCK inhibition or partial deletion of ROCK2 markedly attenuated the development of DACs and associated irregular Ca^{2+} transients induced by increased $[Ca^{2+}]o$ in diabetic cardiomyocytes; 3) the diabetes-induced increases in diastolic Ca^{2+} leak and phosphorylation of the RyR2 were prevented in cardiomyocytes from diabetic ROCK2+/− mice; and 4) the latter was associated with attenuation of the diabetes-induced increased phosphorylation and activation of CaMKII. These data suggest that

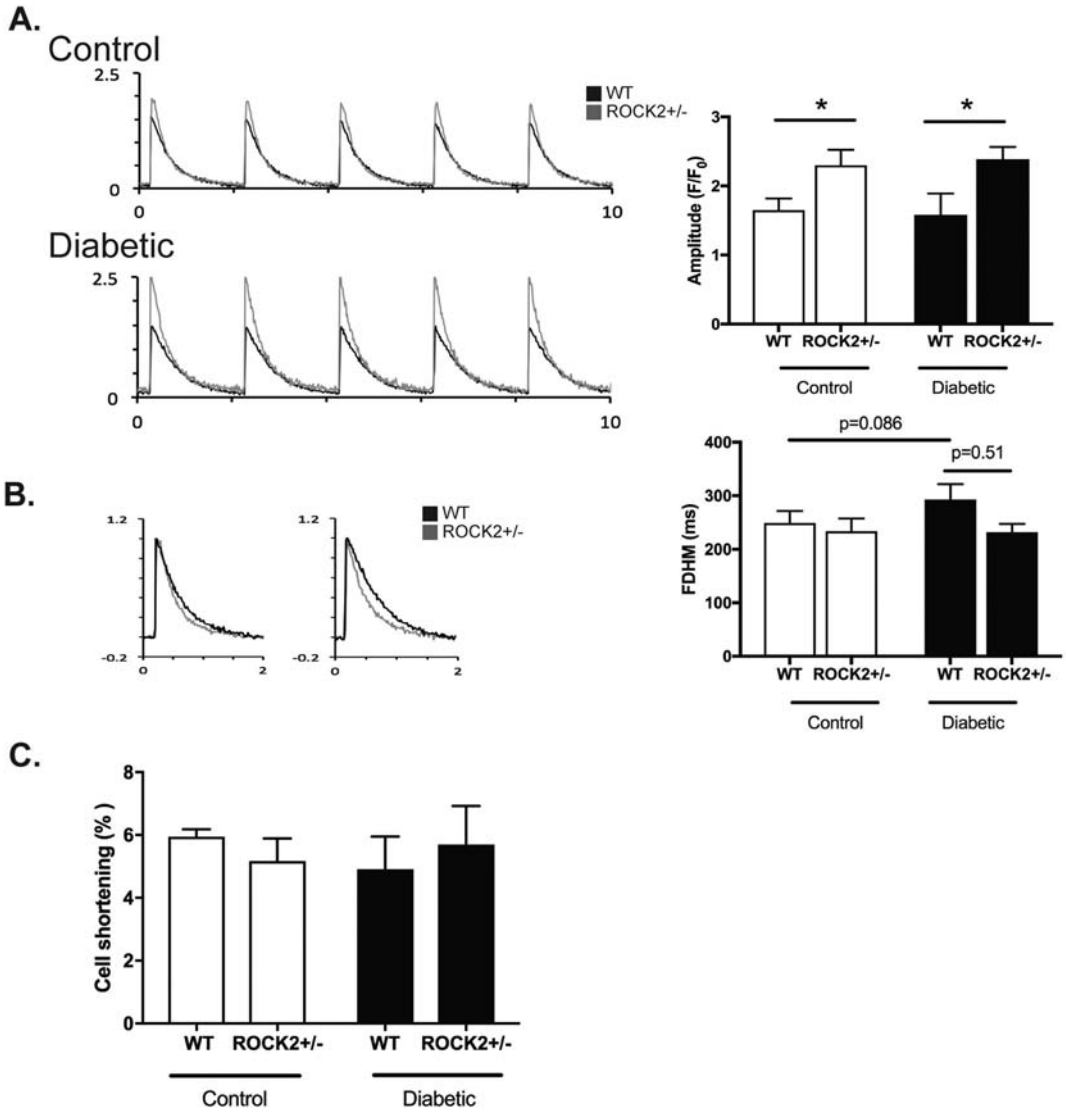


Fig. 2. Effect of partial deletion of ROCK2 on Ca^{2+} transients in cardiomyocytes isolated from control and diabetic mice. Cardiomyocytes isolated from control or diabetic WT and ROCK2+/− mice were incubated at 6 mM Ca^{2+} and stimulated at 0.5 Hz. Fluorescence amplitude (A) and duration to half maximum fluorescence (FDHM; B) were determined. (C) % peak shortening in WT and ROCK2+/− control and diabetic cardiomyocytes. Cardiomyocytes were stimulated at 1 Hz and imaged using a Leica Sp5 confocal microscope equipped with a resonance scanner. *$p < 0.05$ compared to corresponding WT groups; $n = 3$ mice.

hyperactivity of ROCK2 contributes to impaired Ca^{2+} homeostasis in diabetic cardiomyocytes, at least in part by promoting the CaMKII-mediated phosphorylation of the RyR2, increasing the spontaneous release of SR Ca^{2+}.

4.1. Modulation of calcium transient and contraction by ROCK

In this study, cardiomyocytes from diabetic rat hearts displayed only modest changes in the Ca^{2+} transient and in contractile function when perfused with 2 mM Ca^{2+}, with small decreases in peak tension and Ca^{2+} and an increase in time to peak tension, but no significant changes in parameters of relaxation or decay of the Ca^{2+} transient. However, when the Ca^{2+} concentration was increased to 6 mM, the differences in Ca^{2+} transients and contraction between control and diabetic cardiomyocytes became more prominent. The changes detected are similar to those previously reported in cardiomyocytes from diabetic rat hearts, and have been attributed to defective Ca^{2+} handling, largely as a result of impaired SR function [8].

Inhibition of ROCK normalized the time to peak tension, although it had no effect on peak tension, and also improved both the rate of relaxation and time to 50% relaxation at the higher Ca^{2+} concentration. The enhancement of contractile properties by Y-27632 in isolated rat myocytes was associated with improved Ca^{2+} transients, including an elevation of peak Ca^{2+}, a reduction in relaxation time and normalization of the rate of relaxation. This suggests that the acute effect of Y-27632 on contractile function may be, at least partly, through ameliorating the defective Ca^{2+} handling in diabetic rat hearts. This is further supported by the reduction in the propagation delay of the Ca^{2+} transient by Y-27632, since this may result from slower, unsynchronized Ca^{2+} release [24].

However, despite the modest increase in peak Ca^{2+} observed in both 2 and 6 mM Ca^{2+}, peak tension did not change in response to Y-27632 in diabetic cardiomyocytes, which is in contrast to our previous findings in the working heart and *in vivo* [9]. The reason for this is unclear. It may reflect differences between the whole heart and single cardiomyocytes with respect to complexity, mechanical loading conditions or their response to Y-27632. Alternatively, ROCK may affect

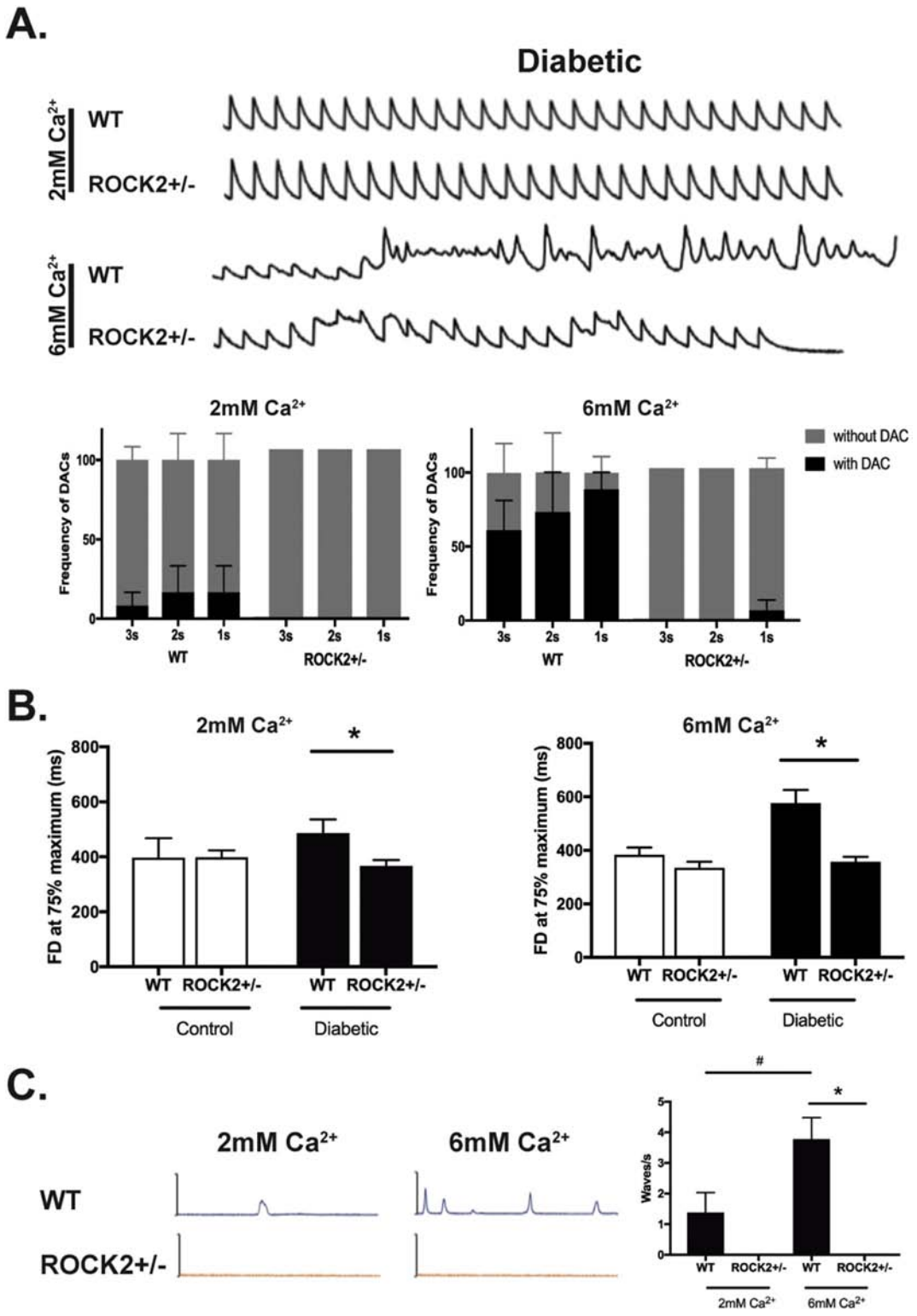


Fig. 3. Partial ROCK2 deletion mitigates Ca^{2+} transient irregularities in diabetic mice. (A) Cardiomyocytes from diabetic WT or ROCK2+/− mice were incubated in 2 mM or 6 mM Ca^{2+} and stimulated at 0.33, 0.5 and 1 Hz. Representative Ca^{2+} transients in diabetic WT and ROCK2+/− cardiomyocytes stimulated at 0.33 Hz (top). Percentage of cells displaying DACs per mouse was determined at each frequency (bottom). (B) The duration to 75% fluorescence was determined in cardiomyocytes from control and diabetic WT and ROCK2+/− mice at 0.5 Hz. (C) Cardiomyocytes from diabetic WT and ROCK2+/− mice were incubated in 2 mM or 6 mM Ca^{2+} and monitored for production of Ca^{2+} waves at rest (no stimulation). *$p < 0.05$ compared to corresponding WT groups, #$p < 0.05$ compared to non-diabetic WT; $n = 4–5$ mice.

excitation-contraction coupling by a mechanism that counteracts any increase in contraction brought about by the increased peak Ca^{2+}, such as through regulation of MYPT phosphorylation [25].

In contrast to our previous observations in diabetic rat hearts, hearts from WT mice with diabetes of 3 months duration did not develop signs of overt systolic or diastolic dysfunction *in vivo*. However,

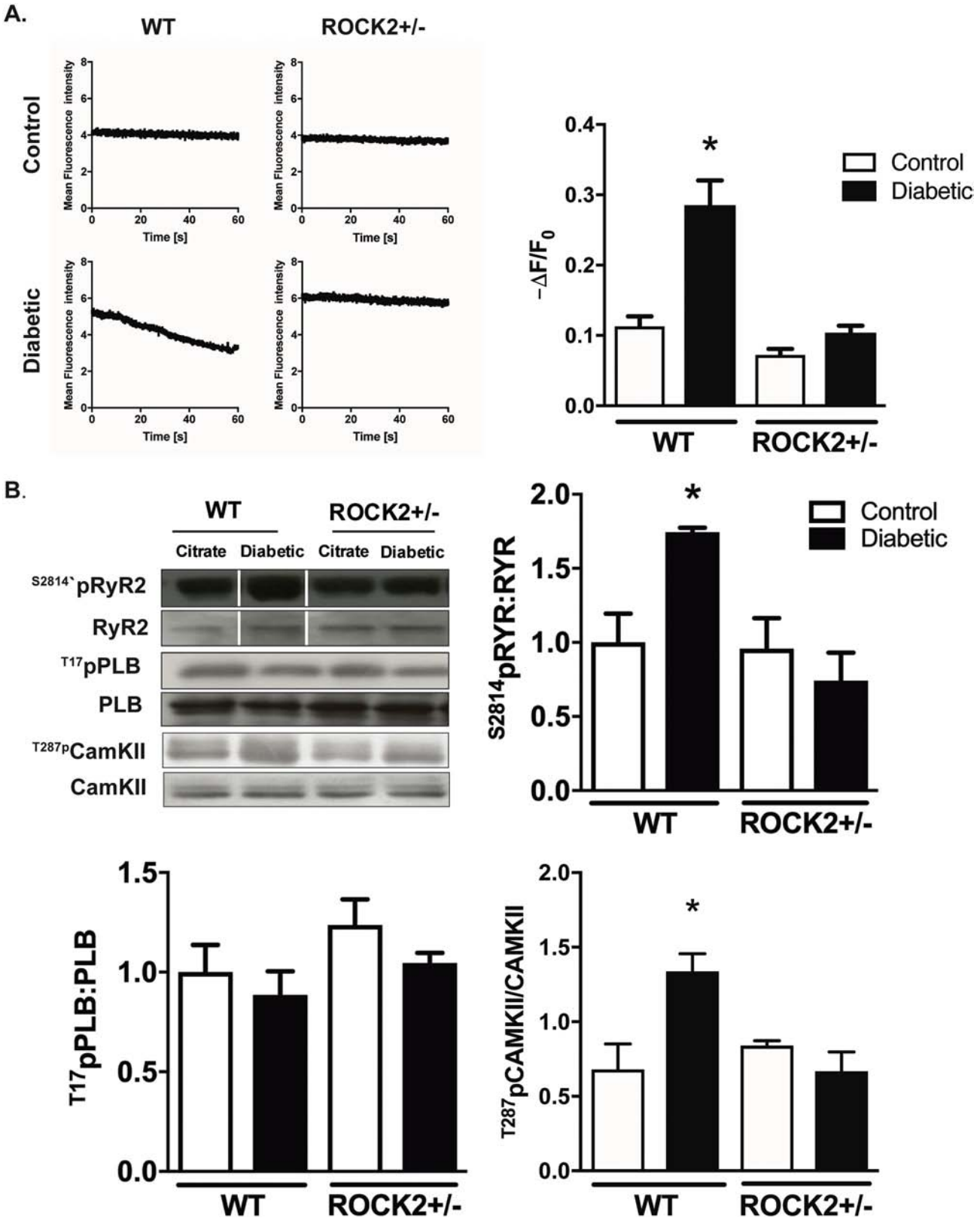


Fig. 4. Partial ROCK2 deletion prevents diabetes-induced diastolic Ca^{2+} leak and increased RyR phosphorylation. (A) SR Ca^{2+} leak was determined in cardiomyocytes isolated from control or diabetic WT and ROCK2+/− mice using the tetracaine method. (B) Representative blots and densitometric analysis of RyR2 S2814, phospholamban T17 and CamKII T287 phosphorylation. Values were normalized to the respective total proteins. *$p < 0.05$ compared to all other groups; $n = 4–5$ mice. White vertical lines in (B) indicate where the representative blots are discontinuous.

speckle-tracking revealed significant endocardial wall motion abnormalities indicating the onset of left ventricular dysfunction in hearts from diabetic WT mice.

These data are consistent with a previous study in diabetic male CD-1 mice (26) and suggest that that onset of overt cardiomyopathy is delayed in this strain of mice compared to rats and to other mouse

strains. No diabetes-induced changes in strain were detected in hearts from diabetic ROCK2+/− mice, suggesting that ROCK2 may contribute to the onset of diabetes-induced ventricular dysfunction [26].

4.2. Effect of ROCK2 inhibition on arrhythmogenic calcium release in diabetic animal models

Diabetes had no detectable effect on Ca^{2+} transients or peak shortening of isolated cardiomyocytes from WT mouse hearts. However, as was found in cardiomyocytes from diabetic rats, diabetic WT mouse cardiomyocytes developed arrhythmic and irregular Ca^{2+} transients under conditions of increased $[Ca^{2+}]o$. Additionally, unstimulated cardiomyocytes from WT but not ROCK2+/− diabetic mice had diastolic Ca^{2+} levels sufficiently high to trigger Ca^{2+} waves at rest. This is consistent with previous reports showing that hearts from diabetic rats and mice are more susceptible to arrhythmias in response to increased intracellular Ca^{2+} produced by elevated $[Ca^{2+}]o$ or isoproterenol [13,15,27,28]. This has been attributed to a diastolic Ca^{2+} leak from the RyR2, which, by increasing diastolic cyoplasmic Ca^{2+}, activates a depolarizing sodium-calcium exchange current that may trigger arrhythmias [8,11,12]. Both acute inhibition of ROCK and partial deletion of ROCK2 prevented the development of the irregular, arrhythmic Ca^{2+} transients, suggesting an important role for ROCK, and in particular ROCK2, in their genesis.

Phosphorylation of the RyR2 at Ser2814 by CaMKII has been shown to increase its sensitivity to Ca^{2+}, resulting in diastolic Ca^{2+} leak [29]. Consistent with this, we found that phosphorylation of the RyR2 in cardiomyocytes from diabetic WT mice was significantly increased, and this was attenuated in myocytes from diabetic ROCK2+/− mice. Furthermore, we found that the phosphorylation of CaMKII, which is associated with its autonomous activation, was also increased in diabetic WT but not ROCK2+/− mice. These data imply that ROCK2 promotes the development of diastolic Ca^{2+} leak in response to increased $[Ca^{2+}]o$ by promoting the activation of CaMKII. That this occurs prior to the detection of overt cardiomyopathy in diabetic mice implies that it is an early event, and that other mechanisms must also contribute to cardiac dysfunction. In agreement with this, acute over-expression of CaMKIIδ has previously been reported to result in diastolic Ca^{2+} leak, leading to reduced SR Ca^{2+} content but not to either reduced twitch contractions or altered Ca^{2+} transients in ventricular myocytes [30]. The molecular mechanism linking ROCK2 to CaMKII activation is currently under investigation, but it is unlikely to involve a direct interaction of the two kinases. Rather, ROCK2 may promote either the oxidation or o-glycosylation of CaMKII, which, at least in the latter case, is accompanied by increased auto-phosphorylation [28].

4.3. Clinical perspective

An increased risk of arrhythmia, in the form of an increased number of supraventricular and ventricular premature beats, has been detected in young type 1 diabetic patients prior to the detection of systolic dysfunction [3]. In addition, diabetic patients are at a significantly greater risk of sudden death due to arrhythmias following myocardial infarction [4,31] and epidemiological studies show that diabetes is an independent risk factor for sudden death [5,32].

To our knowledge, the present study is the first to suggest an anti-arrhythmic effect of ROCK inhibition in a diabetic animal model. Although further investigation is required in order to determine whether this effect is present *in vivo* and can be translated to humans, these data suggest that inhibition of ROCK, and specifically ROCK2, may constitute a novel approach to the treatment of diabetes-associated arrhythmia. Whether the anti-arrhythmic effect of ROCK inhibition extends beyond diabetes to other conditions such as heart failure warrants further investigation.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2019.01.075.

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