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## CaMKII blockade, cardiac conduction, and arrhythmia

We have read the study by Takanari *et al.*<sup>1</sup> published in *Cardiovascular Research* with interest, all the more so given that the reported results are in stark contrast with our own recent study.<sup>2</sup>

The report by Takanari *et al.*<sup>1</sup> explored the effects of acute and chronic inhibition of the Ca<sup>2+</sup>/calmodulin dependent protein kinase II (CaMKII) pathway on ventricular conduction. The results obtained in the chronic setting do not have a counterpart in our own study, and will not be commented here. In the acute setting, the authors used calmodulin blocker W7 (10 µM) in isolated rabbit hearts subjected to cryoablation of the left ventricular (LV) endo- and midmyocardium, leaving only an ~1 mm of viable subepicardial layer. They used optical mapping to estimate the longitudinal (CV<sub>L</sub>) and transverse (CV<sub>T</sub>) conduction velocity (CV) from isochronal activation maps obtained during pacing at a site in the centre of the LV free wall. They found that W7 significantly increased CV<sub>L</sub> and CV<sub>T</sub> by 6.0 and 12.6%, respectively (at the pacing cycle length of 800 ms) and decreased inducibility of ventricular tachycardia/fibrillation (VT/VF) by programmed stimulation. They linked these improvements in conduction to augmented localization of Cx43 in the intercalated disc caused by W7.

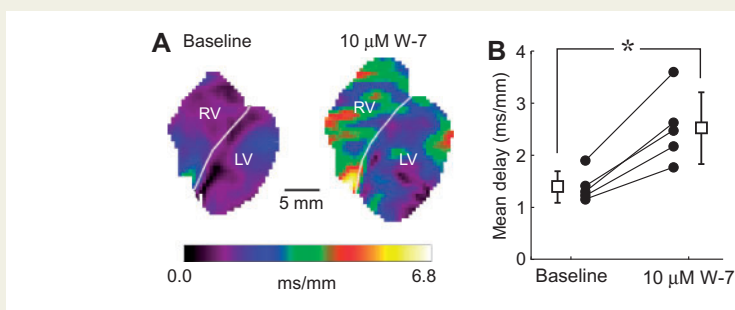
Our recent study using optical mapping in isolated (but otherwise intact) rabbit hearts has demonstrated that CaMKII blockade with 2.75 µM KN93 (KN93 directly targets CaMKII to inhibit its activity) resulted in a marked non-uniform slowing of conduction, with the largest effect occurring in or near the right ventricular (RV) outflow tract (RVOT).<sup>2</sup> The vulnerable region in the RVOT subsequently became a preferred location for 2:1 conduction block, turbulent conduction, and initiation of VF upon subjecting the hearts to global ischemia.<sup>2</sup> Rabbit ventricular myocytes isolated from RVOT exhibited intrinsically lower action potential upstroke velocity (dV/dt<sub>max</sub>) than those isolated from the LV, and KN93 further depressed dV/dt<sub>max</sub> in both locations. In the presence of KN93, RVOT myocytes were much more prone to 2:1 activation failure than LV myocytes after abrupt shortening of the pacing cycle length. We concluded that CaMKII blockade depresses ventricular excitability and is potentially pro-arrhythmic due to exacerbating intrinsically lower excitability in the RVOT, making it vulnerable to

reentrant arrhythmias under conditions causing reduced depolarization reserve.

Assuming that the effects of W7 and KN93 are both due to inhibition of calmodulin/CaMKII pathway, the study by Takanari *et al.*<sup>1</sup> and ours lead to opposite conclusions with regard to the role of this pathway in regulation of cardiac conduction. However, the assumption that W7 and KN93 should have the same effect could be incorrect. Note that whilst KN93 inhibits CaMKII by interfering with its binding to calmodulin,<sup>3</sup> blockade of calmodulin with W-7 will inhibit other downstream calmodulin targets besides CaMKII.<sup>4</sup> In addition, both W7 and KN93 have off-target effects.<sup>5–8</sup> On the other hand, concordance between the effects of W7 and KN93 would strongly suggest that both these effects are due to blocking calmodulin/CaMKII pathway. With this logic in mind, we performed experiments using W7 instead of KN93 in otherwise the same experimental model as in our previous publication.<sup>2</sup> Briefly, continuously perfused (normoxemic) rabbit hearts were exposed to W7 (10 or 20 µM) during sinus rhythm, and periodically subject to ventricular pacing at incremental rates from electrodes placed in the LV and RV. In the absence of mechanical uncouplers, fluorescence emitted by the voltage sensitive dye Di-4-ANEPPS staining the myocardium was collected with an EMCCD camera at a spatial resolution of 64 × 64 pixels (0.56 mm/pix) and frame interval

of 2.06 ms, and the recorded signals used for determining the local conduction delays of the propagating wave fronts. We found that 10 µM W7 induced dramatic slowing of conduction, more pronounced in the RV than in the LV (Figure 1A). The conduction delays increased progressively with time of W7 perfusion, reaching statistical significance at 30 min (Figure 1B). Conduction depression was further enhanced by increasing W7 concentration (10 vs. 20 µM) and/or pacing frequency (3.3 vs. 5.0 vs. 5.9 Hz). Conduction slowing was accompanied by a progressive increase of the excitation threshold and culminated in failure to activate in a 1:1 manner, and/or turbulent conduction, and/or initiation of VT/VF. During the fastest pacing, these abnormal events were absent at baseline (0/5) but were present after 30 min 10 µM W7 perfusion in 4/5 hearts. Perfusion with 20 µM W7 led to sustained episodes of VT/VF maintained by reentrant waves. Time matched control experiments showed complete absence of arrhythmias and no changes in the conduction delays during relevant period of time (mean conduction delay at 0 min (post-equilibration) vs. 30 min: 1.24 ± 0.13 vs. 1.25 ± 0.18 ms/mm, paired Student's *t*-test *P* = 0.88, *n* = 4). Thus, we found that the effects of W7 are indeed concordant to the effects of KN93 we observed previously in Ref. 2.

Despite using the same drug and the same species, there remain methodological differences



**Figure 1** Calmodulin blocker W7 depresses cardiac conduction in intact rabbit hearts. (A) Distribution of local conduction delay values in the imaged anterior surface of both ventricles at baseline (left) and after 30-min perfusion with 10 µM W7 (right). Local delays (the inverse of the local conduction velocities) were computed as the magnitude of the gradient of the activation time. Hearts were paced simultaneously from two electrodes located at the lateral epicardial surface of each ventricle at a cycle length of 300 ms. White labels indicate the LV and RV location, and the white lines indicate the boundary between the chambers. (B). Mean conduction delays computed over the entire mapped surface at baseline and after 30 min perfusion with 10 µM W7: solid circles represent values for individual experiments; open square symbols and bars represent average and SD. \**P* = 0.00352 vs. baseline, paired Student's *t*-test (*n* = 5 hearts).

between the Takanari's study<sup>1</sup> and our own<sup>2</sup> which could explain the discrepant outcomes. Our model might be more physiological not involving endomidmyocardial cryoablation and electromechanical uncoupler/non-specific phosphatase 2, 3-butandione monoxime (used by Takanari et al. at unusually high concentration of 100 mM). In addition, we analyzed local delays (inverse of CV) in all mapped RV and LV locations, not only in a selected region around the pacing electrode in the LV. This enabled us to find the epicenter of the depressant effect, located in the RV. Yet, even if we limited our analysis to the LV, we would still end up with results opposite to those by Takanari et al.<sup>1</sup>

Although various studies converge in considering CaMKII as a principle regulator of cardiac excitability, there is disagreement as to how this occurs. This controversy is particularly evident in single cell studies investigating how CaMKII regulates  $I_{Na}$ , with some suggesting that CaMKII activity favors an overall  $I_{Na}$  upregulation,<sup>9,10</sup> whilst others argue that in fact CaMKII activity promotes an overall  $I_{Na}$  downregulation.<sup>11</sup> Consideration of Takanari's study<sup>1</sup> and our own<sup>2</sup> clearly shows that this divergence has translated to the intact tissue level. In our view, the similarity of (adverse) effects exerted by W7 and KN93 on ventricular conduction strongly reinforces the notion that constitutive activity of the calmodulin/CaMKII pathway is necessary for normal ventricular excitation and conduction.<sup>2</sup> Clearly, sorting this matter is important given that ineffective regulation of conduction can potentially lead to life-threatening arrhythmia. In the light of our observation that calmodulin/CaMKII blockade partially mimicked Brugada syndrome,<sup>2</sup> patients having Brugada syndrome or other conduction diseases can be at enhanced risk of VF if treated with CaMKII blockers. Other conditions reducing depolarization reserve can also adversely interact with calmodulin/CaMKII blockade. It is important to ascertain the effect of calmodulin/CaMKII blockade on conduction in both normal and diseased heart before CaMKII blockers can be translated into clinical practice.

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## Response to the letter from Warren et al.

With special attention, we have read the letter from Dr Warren and Dr Zaitsev.

In their letter, they react on our recent publication in *Cardiovascular Research*,<sup>1</sup> in which we used four different experimental *in vitro* and *in vivo* models to test the acute and chronic effects of inhibiting the calmodulin/calmodulin-dependent protein kinase II (CaM/CaMKII) pathway using different

pharmacological and genetic approaches. Outcome of our study showed that acute inhibition of this pathway: (i) was anti-arrhythmic in a rabbit heart model of pacing-induced arrhythmias, (ii) improved intercellular communication in a model of cultured neonatal ventricular rat cardiomyocytes, (iii) augmented the localization of gap junction protein Cx43 in the intercalated disc of isolated and cultured adult dog cardiomyocytes and in rabbit heart, while in a chronic setting: (iv) augmented conduction velocity of impulse propagation at baseline in a genetically engineered mouse model of chronic CaMKII inhibition, preserved this conduction velocity upon pressure overload although in this model arrhythmias could not be prevented presumably due to the absence to prevent deposition of fibrosis.

In their letter, including data of additionally performed experiments following their recent study as published in the *American Journal of Physiology*,<sup>2</sup> they state that in the experiments they performed, acute application of 10–20  $\mu$ M W7 resulted in conduction delay or conduction block and proarrhythmia in isolated rabbit hearts, which was intrinsically opposite to our outcomes.<sup>1</sup> This dose is slightly higher to the one we used in our study, although we have also tested higher concentrations of W7 (20–50  $\mu$ M), which seemed to depolarize the membrane potential, in accordance with previous work,<sup>3</sup> reduced excitability of the heart, and consequently impaired conduction properties of the perfused rabbit hearts. Excitability and conductivity of the heart tissue strongly relies on the sodium current ( $I_{Na}$ ) and inward rectifier potassium current ( $I_{K1}$ ). The effect of CaM/CaMKII inhibition on  $I_{Na}$  is still controversial as Warren et al. described in their letter,<sup>4,5</sup> and the effect of CaM/CaMKII on  $I_{K1}$  is also still disputable. It has been reported that inhibition of CaM or CaMKII might affect  $I_{K1}$  in canine cardiomyocytes.<sup>6</sup> More recent literature also suggested the influence of CaMKII on  $I_{K1}$  in canine and human heart.<sup>7</sup> However, a recent study showed that there was no acute effect of W7 on  $I_{K1}$  in isolated neonatal rat cardiomyocytes.<sup>8</sup>

It seems that the effect of CaM inhibition on  $I_{Na}$  and  $I_{K1}$  differs according to the experimental conditions. It also appeared that acute W7 had a biphasic action on conduction; it might accelerate conduction at the lower dose, and on the other side it might also impair the conduction properties at the higher dose. We suppose that such biphasic action of CaM inhibition might be resulting from a balance between antiarrhythmic effects (augmentation of conduction properties, such as an increase in gap junctional conductance) and proarrhythmic effects (impairment of conduction properties, such as reduction of  $I_{Na}$  and  $I_{K1}$ ). We at that time concluded that a high amount of W7 might impair the excitability via the inhibition of  $I_{K1}$  and/or  $I_{Na}$ .

In our study, we generally focused on the effect of W7 on intercellular communication and conduction velocity, and more specifically within the rabbit model on those aspects in the setting of