Relevance of calmodulin/CaMKII activation for arrhythmogenesis in the AV block dog

Vincent J.A. Bourgonje, Msc,* Marieke Schoenmakers, MD, PhD,† Jet D.M. Beekman,* Roel van der Nagel, Bsc,* Marien J.C. Houtman, Bsc,* Lars F. Miedema, Bsc,* Gudrun Antoons, PhD,‡ Karin Sipido, MD, PhD,§ Leon J. de Windt, PhD,*† Toon A.B. van Veen, PhD,* Marc A. Vos, PhD*

From the *Department of Medical Physiology, Division Heart & Lungs, University Medical Center Utrecht, Utrecht, The Netherlands, †Department of Cardiology, Cardiovascular Research Institute Maastricht, University Maastricht, Maastricht, The Netherlands, and §Laboratory of Experiment Cardiology, KU Leuven, Leuven, Belgium.

BACKGROUND The calcium-dependent signaling molecules calcineurin and calcium/calmodulin–dependent protein kinase II (CaMKII) both have been linked to decompensated hypertrophy and arrhythmias. CaMKII is also believed to be involved in acute modulation of ion channels.

OBJECTIVE The purpose of this study was to determine the role of calcineurin and CaMKII in a dog model of compensated hypertrophy and a long QT phenotype.

METHODS AV block was created in dogs to induce ventricular remodeling, including enhanced susceptibility to dofetilde-induced torsades de pointes arrhythmias. Dogs were treated with cyclosporin A for 3 weeks, which reduced calcineurin activity, as determined by mRNA expression levels of regulator of calcineurin 1 exon 4, but which was unable to prevent structural, contractile, or electrical remodeling and arrhythmias. Biopsies were taken before and at 2 or 9 weeks after AV block. Western blots were performed against phosphorylated and total CaMKII, phospholamban, Akt, and histone deacetylase 4 (HDAC4).

RESULTS Chronic AV block showed an increase in Akt, CaMKII and phospholamban phosphorylation levels, but HDAC4 phosphorylation remained unaltered. Dofetilde-induced torsades de pointes in vivo and early afterdepolarizations in cardiomyocytes, and increased [Ca2+]i, and CaMKII autophosphorylation. Both W-7 and KN-93 treatment counteracted this.

CONCLUSION The calcineurin pathway seems not to be involved in long-term cardiac remodeling of the chronic AV block dog. Although CaMKII is chronically activated, this does not translate to HDAC4 phosphorylation. However, acute CaMKII overactivation is able to initiate arrhythmias based on triggered activity.

KEYWORDS Arrhythmias; Calcineurin; CaMKII; Chronic AV block dog; Ventricular remodeling

ABBREVIATIONS ANP = atrial natriuretic peptide; APD = action potential duration; CaMKII = calcium/calmodulin–dependent protein kinase II; CAVB = chronic AV block; CNa = calcineurin-A; CSA = cyclosporin A; EAD = early afterdepolarization; HDAC4 = histone deacetylase 4; LV = left ventricle; MAP = monophasic action potential; MEF2 = myocyte enhancer factor 2; PCR = polymerase chain reaction; Rcan1–4 = regulator of calcineurin 1 exon 4; ROS = reactive oxygen species; STV = short-term variability; Tdp = torsades de pointes (Heart Rhythm 2012;xx:xxx) © 2012 Heart Rhythm Society. All rights reserved.

Introduction

Cardiac remodeling can result in a compensated or a decompensated (heart failure) phenotype. In both conditions, remodeling predisposes for arrhythmias.

In the failing human heart, expression of the calcium-sensitive signaling molecules calcium/calmodulin–dependent protein kinase II (CaMKII)1,2 and calcineurin-A (CNa) is increased.3 Also, in mice overexpressing CNa4 or CaMKII,5,6 heart failure and arrhythmias are present. In contrast, the involvement of CaMKII or calcineurin in compensated hypertrophy is less established, and, in exercised mice, calcineurin/nuclear factor of activated T-cells (NFAT) was not activated,7 whereas CaMKII knockout mice subjected to pressure overload had preserved cardiac function.8

Acutely activated CaMKII can also directly phosphorylate ion channels, modulate excitation and contraction, and induce arrhythmias.9 Therefore, CaMKII-induced proarrhythmic modulation can be subdivided between acute and chronic processes,10 which both can be investigated through pharmacologic intervention. Information concerning acute modulating effects of calcineurin on electrophysiology is currently not available.

Known stressors for increased activation/expression of CaMKII and calcineurin are present in the chronic AV block (CAVB) dog model, including elevated calcium levels. Other remodeling processes, such as prolonged QT interval, dispersed action potential duration (APD), and...
biventricular hypertrophy, also have been described, resulting in a high susceptibility for torsades de pointes (TdP) arrhythmias after acute administration of the IkR blocker dofetilide. In line with this, isolated CAVB cardiomyocytes are susceptible to early afterdepolarizations (EADs) after dofetilide infusion.

In the CAVB dog, remodeling results in preserved ventricular function. We hypothesized that in the CAVB dog model, CaMKII and calcineurin, mirroring their role in heart failure, would differentially contribute to cardiac remodeling and arrhythmogenesis, which was assessed by pharmacologic modulation of CaMKII and calcineurin, in vivo and in vitro.

Methods

Twenty-two dogs (25 ± 4 kg, 63% female) were used in this study using 6 different protocols (A–F). Three experimental procedures (protocols A, B, and C) were designed to reveal the role of calcineurin and CaMKII in AV block–induced ventricular remodeling and arrhythmias, whereas cellular confirmation of these data was the central goal of protocols D, E, and F.

Animal handling was in accordance with the European Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Community Directive #86/609/CEE). The Committee for Experiments on Animals (DEC) of Maastricht (part A) or Utrecht University (parts B–C), The Netherlands, approved the experiments. Experiments were performed with the animals under general anesthesia, and AV block was induced by radiofrequency ablation. Specific details concerning anesthesia, AV nodal ablation, TdP characteristics, and instrumentation can be found in the Online Supplemental Methods. In this study, the following drugs were used:

- Dofetilide: a specific IkR blocker used to induce arrhythmias in this model (protocols B and D)
- W-7: a calmodulin inhibitor, and thus an upstream blocker of CaMKII
- KN93: a direct CaMKII blocker, used in vitro as an acute antiarrhythmic drug (protocol D)
- Cyclosporin A (CSA): a calcineurin inhibitor, used chronically in vivo (protocol A) and acutely in vitro (protocol D)
- Isoproterenol: adrenergic agonist used as a positive control for CaMKII activation in protocol F

Protocol A: In vivo CSA experiment

Twelve adult mongrel dogs were randomly divided: 5 received CSA (10 mg/kg bid) treatment from 1 week before AV block until 2 weeks after (Figure 1A), and 7 served as a control group. Animals were tested twice: (1) at sinus rhythm and directly after AV block, and (2) 2 weeks later (CAVB2). In addition to regular measurements, left ventricular (LV) pressure (Sentron, Roden, The Netherlands) and +dP/dt\textsuperscript{11} were determined. At CAVB2, an arrhythmia challenge was performed using dofetilide (25 μg/kg/5 minutes, or until first TdP). Afterward, dogs were sacrificed to obtain heart weight, biopsy sample, and cardiomyocytes.

The effectiveness of CSA as a calcineurin inhibitor was confirmed using quantitative polymerase chain reaction (PCR) on regulator of calcineurin 1 exon 4 (Rcan1-4), a gene downstream of calcineurin (for protocol, see Online Supplemental Methods).

Protocol B: W-7 in vivo antiarrhythmia experiment

At CAVB (≥5 weeks), W-7 (50 μmol/kg/5\textsuperscript{12}) (Biomol) was given as an antiarrhythmic drug (Figure 2A) against TdP \textsuperscript{2} (N = 7). The number of TdPs was quantified at 5-minute intervals. Short-term variability (STV) was determined over 30 consecutive beats using the following formula: STV = Σ1.30|Dn – Dn–1|/(30 × √2), where D = LV MAPD\textsuperscript{90}.

Protocol C: Ventricular biopsies

Needle biopsies were taken (N = 6 from protocol B and N = 3 additional CAVB dogs) and used for Western blot or quantitative PCR. For the complete protocol, see the Online Supplemental Methods.

Protocol D: Patch clamp on cardiomyocytes

Hearts were excised and cardiomyocytes enzymatically dissociated, as described previously.\textsuperscript{14} Nineteen cells from 6 dogs from protocol B were used, of which 9 cells were treated with KN-93, 6 with W-7, and 4 with cyclosporin. Cells were patch clamped and the experiments conducted (Figure 4A and Online Supplemental Methods).

Protocol E: [Ca\textsuperscript{2+}]\textsubscript{i} during dofetilide treatment

In cardiomyocytes (N = 13 from 6 dogs of protocol B), calcium transients were determined at baseline and with dofetilide (for examples, see Figures 5A and 5B). For the full protocol, see the Online Supplemental Methods.

Protocol F: Acute treatment of cardiomyocytes with dofetilide and W-7

Cardiomyocytes (protocol B) were transferred to culture dishes (±50% confluency) and incubated at 37°C for 10 minutes in normal Tyroe’s solution with 1 μM dofetilide, 1 μM dofetilide + 50 μM W-7, or 1 μM isoproterenol (Figure 5F). After incubation, cells were lysed and frozen. CaMKII autophosphorylation levels were determined by Western blotting (see protocol C).

Statistical analysis

All data are presented as mean ± SD. Details of the tests can be found in the Online Supplemental Methods.

Results

Part A: Long-term proarrhythmic remodeling is not dependent on calcineurin

Treatment with CSA prior to AV block increased heart rate (106 ± 21 bpm vs 149 ± 22 bpm, P < .01) but no other electrical parameter. Therefore, acute AV block parameters were set at 100%.

Blockade of calcineurin neither prevented electrical (QTc, Figure 1B) nor mechanical remodeling (LV dP/dT, Figure 1C) nor influenced heart
weight (9 g/kg). Arrhythmogenicity after dofetilide challenge also remained similar (TdP incidence 5/7 vs 4/5 dogs). Rcan1-4, a downstream target of calcineurin (Figure 1D), was used as a control for cyclosporin-induced calcineurin inhibition. As shown in Figure 1E, Rcan1-4 was detectable in untreated dogs, but amounts fell below detection thresholds with cyclosporin treatment.

In addition, no increase in Rcan1-4 expression during the remodeling process in untreated CAVB dogs was observed (Figure 1E), indicating that calcineurin activity was not altered between sinus rhythm and 2 (CAVB2) or 9 weeks of AV block (CAVB9+).

Part B: In vivo CaMKII inhibition is antiarrhythmic

Dofetilide prolonged QT and induced TdP (Figure 2B, top panel). W-7 suppressed almost all TdPs (Figure 2B, bottom panel). Quantification of this effect is illustrated in Figure 2C. The antiarrhythmic effect of W-7 was accompanied by LV monophasic action potential (MAP) duration shortening (Figure 2D) but no effect on PP (baseline 582 ± 131 ms, dofetilide 703 ± 225 ms, and after W-7 727 ± 158 ms).

Part C: CaMKII pathway is chronically activated in CAVB dog but is truncated at histone deacetylase 4

Total CaMKII levels (CaMKII-T) were not altered during CAVB (from 1 ± 0.1 at sinus rhythm, to 0.8 ± 1 at CAVB2, to 1 ± 0.2 at CAVB9+), but autophosphorylation was increased (Figure 3A). Phospholamban, a direct target of CaMKII at Thr17, was also more phosphorylated (Figure 3B). Next we assessed the phosphorylation status of histone deacetylase 4 (HDAC4), which is a known target of CaMKII and is involved in activating a myocyte enhancer factor 2 (MEF2C)–mediated maladaptive gene expression profile. In our model of compensated hypertrophy, phosphorylation of HDAC4 was completely identical between sinus rhythm and CAVB9 (Figure 3C). In contrast, a marker of physiologic hypertrophy, Akt, was increased (Figure 3D).

Part D: In vitro CaMKII inhibition is antiarrhythmic, but calcineurin inhibition is not

Electrically stimulated and dofetilide-treated cardiomyocytes showed action potential prolongation (Figure 4B), increased STV (Figure 4C), and EADs (Figure 4D). Once W-7 was cotransfused, EADs disappeared (in 6/6 cells), and...
APD and STV decreased significantly (Figures 4B and 4C). KN-93 showed comparable results (EADs disappeared in 8/9 cells), whereas calcineurin inhibition by CSA was not antiarrhythmic (Figure 4D). Results for every individual cell can be found in Online Supplemental Figure 1.

Parts E+F: Dofetilide treatment increases the Ca transient and activates CaMKII, an effect suppressed by W-7
Dofetilide increased calcium in CAVB cardiomyocytes, both at diastole (Figure 5C) and systole (Figure 5D), as well as calcium amplitude (Figure 5E). Dofetilide treatment also increased CaMKII autophosphorylation, comparable to the effect induced by the positive control isoproterenol. Co-treatment with W-7 was able to suppress this enhanced CaMKII phosphorylation completely (Figure 5G).

Discussion
Cardiac remodeling in the CAVB dog can be subdivided into a number of processes. First, electrical adaptations lead to a longer APD, increased temporal dispersion of repolarization (STV), and increased arrhythmogeneity. Second, cardiac output is preserved by compensatory contractile remodeling. Third, biventricular hypertrophy develops. We previously demonstrated that structural remodeling did not participate dominantly because, in the absence of hypertrophy, TdP could be induced.

In the present study, we aimed to assess the potential contribution of CaMKII and CnA in this remodeling process, given the knowledge that both show increased and maladaptive activity under conditions of heart failure. Moreover, the interaction between CaMKII and CnA is subject to discussion. Different relationships have been suggested, ranging from a mechanism in which CaMKII negatively controls the downstream activity of CnA, to cross-talk, in which both contribute to electrical remodeling. It has even been postulated that CaMKII is downstream of CnA, as long-term CaMKII inhibition reduced arrhythmogenesis and improved contractile function in CnA overexpressing mice.

In this model of compensated hypertrophy with a high susceptibility for TdP, we demonstrated that CSA treatment could prevent neither ventricular remodeling nor arrhythmogenesis, nor did CAVB induce activation of CnA, as Rcan1-4 expression remained unaltered (Figure 1). Chronically, there was increased phosphorylation of CaMKII and its target phospholamban. However, HDAC4 phosphorylation...
tion remained unchanged, whereas phosphorylation of Akt was increased (Figure 3). Dofetilide administration increased Ca\textsuperscript{2+}/H\textsubscript{11001}\textsuperscript{+} transients (Figure 5), CaMKII activity (Figure 3), APD, and STV, and induced EADs (Figure 4). In vivo, dofetilide repeatedly induced TdP (Figure 2), which could be antagonized by W–7. KN–93 and W–7, but not CSA, Figure 3 Phosphorylation levels of CaMKII, phospholamban, HDAC4, and Akt during cardiac remodeling in CAVB dog. A: Representative Western blots of phosphorylated CaMKII (CaMKII-P) and total CaMKII (CaMKII-T). *P < .05 vs sinus rhythm. B: Idem, but now on phosphorylated phospholamban (PLB-P) and total phospholamban (PLB-T). C: Idem, but now on phosphorylated HDAC4 (HDAC4-P) and on total protein Ponceau staining (Ponceau). D: Idem, but now on phosphorylated Akt (Akt-P) and total Akt (Akt-T).

Figure 4 CaMKII inhibition is antiarrhythmic in vitro and decreases APD and STV. A: Quantification of electrical parameters, with the protocol and a representative example. APD\textsubscript{90} (B) and STV (C) were determined in 10 consecutive beats. D: EADs scored. NP = not possible *P < .05 dofetilide vs dofetilide + antidote. \( ^{*}p < .05 \) baseline vs dofetilide.
also were antiarrhythmic in vitro (Figure 4). Thus, using in vivo and in vitro experiments, we conclude the following: (1) the CnA pathway is not involved in cardiac remodeling of the CAVB dog, (2) the activated CaMKII pathway does not lead to HDAC4 activation, and (3) acutely increased CaMKII activity is able to initiate arrhythmias. The role of CaMKII and CnA in the CAVB dog is summarized in Figure 6.

CnA activity in the CAVB dog

The CnA pathway has been implicated in the development of pathological hypertrophy but not in exercise-induced physiological (compensated) hypertrophy. Here we showed that remodeling upon AV block cannot be prevented by CnA inhibition and also does not result in increased expression levels of Rcan1-4, a target of CnA. Moreover, we demonstrated that CSA cannot suppress EADs in vitro. These results are in contrast with those of a study by Schreiner et al., in which CSA pretreatment appeared to be effective in diminishing hypertrophy in CAVB dogs. However, a 43% mortality rate was noted in their control group, suggesting that heart failure may be part of the remodeling. In our study, mortality after creation of AV block was <5%. Absence of involvement of CnA is further stressed by the notion that activation of the pathway causes reexpression of the voltage-gated T-type Ca2+ channel. However, this channel is not detectable in our CAVB dog model. Based upon our current data, with respect to a potential role of CnA in remodeling of the CAVB heart, we conclude that CnA is not involved with respect to both electrophysiologic and structural remodeling.

CaMKII activity and compensated hypertrophy in the CAVB dog

Previous studies have shown that chronic activation of CaMKII by (1) constitutive overexpression in mice or (2) increased expression in failing human myocardium eventually will result in heart failure, dilation, arrhythmogenesis, and sudden death. More recently, Ling et al. concluded that chronic CaMKII activation and altered gene expression could be restricted to diluted cardiomyopathy in which CaMKII activation leads to a number of adaptations, including altered Ca2+ handling, more apoptosis, and fibrosis.

That the calmodulin/CaMKII pathway is chronically activated in the CAVB dog seems contradictory in this respect, because the compensated hypertrophy in the CAVB dog...
does not deteriorate into heart failure. One explanation is the intensity of CaMKII signaling. In the CAVB dog only activity is increased, whereas in heart failure models both activity and expression are greater. Importantly, in the CAVB model, increased activation without increased expression of CaMKII did not result in higher HDAC4 phosphorylation. Activation of HDAC4 leads to MEF2-induced expression of pathologic hypertrophy genes, such as atrial natriuretic peptide (ANP), brain natriuretic peptide, and \(\beta\)-myosin heavy chain.\(^{16,17}\) Backs et al\(^{15}\) previously showed that this maladaptive remodeling can be blocked by a phosphorylation-resistant HDAC4 mutant. The lack of CaMKII-induced activation of the HDAC4/MEF2/gene expression pathway is supported by the decoupling of CaMKII activity and ANP levels in the CAVB dog. At 9 weeks of remodeling, ANP plasma levels returned to baseline,\(^{19}\) whereas CaMKII was still chronically activated (shown in this study). Chronic inhibition of CaMKII is known to inhibit deterioration into heart failure but not hypertrophy.\(^{8}\) Apparently, the signaling downstream of CaMKII differs between compensated and decompensated so that, in the compensated state, the HDAC4/MEF2 driven gene expression pattern is prohibited, which potentially explains the absence of deterioration into dilated cardiomyopathy. These observations are supported by the increased levels of phosphorylated Akt (Figure 3D), which are linked to physiologic hypertrophy rather than heart failure.

**CaMKII activity and arrhythmogenesis in the CAVB dog**

We could not confirm CnA activation in this model, while CaMKII was chronically phosphorylated. This finding implies that a previously suggested link where CaMKII activation appears downstream from CnA, although probably present, is not functional after AV block in this model.

Known CaMKII activators are calcium/calmodulin and/or reactive oxygen species (ROS). The first can be distinguished in diastolic cytoplasmic Ca\(^{2+}\) levels, systolic levels, and nuclear calcium, whereas the second can be activated by angiotensin II.\(^{29}\) In the CAVB dog, the Ca\(^{2+}\) transient is chronically increased in amplitude and duration, indicating elevated systolic calcium levels, whereas the diastolic Ca\(^{2+}\) concentration is equal to normal physiologic states as seen in dogs in sinus rhythm.\(^{31}\) The involvement of nuclear calcium is unknown. Activation through ROS cannot be completely excluded. Levels of its natural ligand AngII are only transiently elevated and return to baseline at 5 weeks of remodeling,\(^{32}\) but aldosterone levels, also identified as a source of ROS-induced CaMKII activation,\(^{33}\) do not completely return to baseline values.\(^{32}\)

On the other hand, ROS are known to directly activate CaMKII and induce EADs in a calmodulin-independent way.\(^{34}\) In that respect, the antiarrhythmic effect of the calmodulin inhibitor W-7 suggests that alterations in calcium handling are the most important in this model. In addition, dofetilide administration did increase systolic and diastolic [Ca\(^{2+}\)]\(_{i}\) (Figure 5).

**Potential mechanisms underlying proarrhythmic CaMKII activity**

The dofetilide-induced CaMKII activation is modest (Figure 6) but provided an additional contribution to the already more activated CaMKII due to remodeling in CAVB. In another CAVB model, the CAVB rabbit, chronic CaMKII activity is also present,\(^{35,36}\) but spontaneous arrhythmias develop.\(^{35}\) In that respect, dofetilide-induced overactivation of CaMKII brings the CAVB dog closer to the rabbit model.
Because dofetilide increased CaMKII activity and associated arrhythmias within minutes in our experiments, and its inhibition is antiarrhythmic within minutes as well, the logical targets might be directly modulated by CaMKII.

A previous study in the CAVB model discovered that triggered activity, but not reentry, is the leading cause of arrhythmias in this model.37 This leaves a limited number of remaining possibilities, namely, the modulatory effect on potassium, late sodium, and calcium currents. Thus, a decrease in APD/QT or control of [Ca], are the key antiarrhythmic actions. Blockade of CaMKII will decrease the activity of potassium channels,10 which is opposite of being antiarrhythmic, whereas under these conditions both the CaMKII effect on increasing sodium late38 or the L-type calcium channel is antiarrhythmic in the CAVB dog.39 Second, a window current exists in CAVB cardiomyocytes, with altered inactivation,27 which is in concordance with changes observed in the CAVB rabbit, in which altered L-type calcium channel inactivation could be prevented by CaMKII inhibition.40

It is difficult to separate effects on APD from those on [Ca] handling because they affect each other. In our experiments, W-7 shortened APD, putting into question a dominant effect on [Ca] handling. This in contrast to other investigations, who showed in a rabbit model of long QT that W-7 can reduce action potential variability and arrhythmias without APD shortening. The latter is a strong suggestion of involvement of CaMKII inhibition.40 In order to unravel the exact mechanisms of how CaMKII affects ion currents and Ca handling, future studies are required.

Study limitations
Because of limited availability, KN-93 was used only for in vitro assays. For the same reasons, we limited our W-7 in vivo experiments to a suppression (and not prevention) protocol only.

We acknowledge that KN-93 potentially induces effects beyond CaMKII inhibition, for example, on the L-type calcium channel,41 and that W-7 is known to be a calmodulin inhibitor. However, other drugs that specifically inhibit CaMKII are still not available, and peptides such as AIP, although specific, are not useful for suppression experiments. Similarly, CSA, the well-known inhibitor of CnA, exerts effects on other targets as well. For that reason, although our data show that CnA is not activated, we cannot exclude that other CSA-sensitive and unidentified targets contributed to the effects observed after the intervention with CSA.

Conclusion
In the CAVB model of compensated hypertrophy, CaMKII is chronically phosphorylated, but without activation of the HDAC4/MEF2 and calcineurin pathways. Nonetheless, a further increase in CaMKII phosphorylation induced by a pharmacologic challenge initiated arrhythmias based on triggered activity.

References
Supplemental methods

Anesthesia, AV nodal ablation, and drugs used

Premedication consisted of 0.5 mg/kg methadone, 0.5 mg/kg acempramide, and 0.02 mg/kg atropine IM. Anesthesia was induced with pentobarbital (Nembutal 25 mg/kg IV) or thiopental (15 mg/kg) and maintained by isoflurane (1.5% in O2 and N2O, 1:2). Lack of pupillary response was checked to assess adequate anesthesia. In addition, heart and respiratory rates were constantly monitored. AV block was induced by radiofrequency ablation, which led to an acute drop in heart rate from 117 ± 28 bpm to 58 ± 14 bpm. During all in vivo experiments, a 6-lead ECG and two monophasic action potentials (EP Technologies, Sunnyvale, CA) were recorded from the endocardium in both the left ventricle (LV) and the right ventricle.

Tdp was defined as 5 consecutive extra beats, and a dog was considered “inducible” if 3 or more Tdp arrhythmias were seen in the first 10 minutes after start of infusion. If necessary (>15 seconds), the animals were defibrillated through patches that had been placed in advance.

Protocol C: Ventricular biopsies

During open-chest surgery, 3 simultaneous LV free wall transmural biopsial samples were taken using a 14G automatic biopsy needle (Accut, TSK Laboratory, Japan), and subsequently snap-frozen in liquid nitrogen and stored at −80°C. Biopsy samples were taken serially either at sinus rhythm and 2 weeks of AV block (3 dogs) or at sinus rhythm and 9+ weeks of AV block (3 dogs) to isolate protein and/or RNA (TRizol reagent) for subsequent Western blots or quantitative PCR. In another 3 animals, biopsy samples were taken only at 9+ weeks of AV block.

Quantitative PCR with Rcan1-4 primers

Expression levels of Rcan1-4 were determined by quantitative PCR (Bio-Rad iCycler machine), using iQ SYBR Green Supermix (Bio-Rad).

GAPDH was used for normalization. For GAPDH the following primers were used: forward 5’-AGTCAAGGC-TGAGGACGGAAACT-3’ and reverse 5’-TCCACAACAT-ACTTGCACCAGCA-3’. The Rcan1-4 primers used were as follows: forward 5’-ACCGTTAGGCTTTCACTG-3’ and reverse 5’-TGTTTGCCACACACGCAATC-3’.

Western blots

Biopsy samples were pulverized using a precooled steel mortar and subsequently lysed with RIPA lysis buffer. Isolated protein was used for SDS-PAGE and subsequent Western blotting. Chemiluminescence was performed using the ECL kit from Amersham. The following antibodies were used: anti-CaMKII (Abcam), anti-phospho-CaMKII (Affinity Bioreagents or Santacruz), anti-phospholamban (Thermo-Scientific), anti-phosphorylated (threonine 17) phospholamban (Santacruz), anti-phosphorylated HDAC4 (Abcam), anti-Akt (Cell Signaling), anti-phosphorylated Akt (Cell Signaling).

Protocol D: Patch clamp experiments on LV isolated cardiomyocytes

Hearts were quickly excised, and single cardiomyocytes were enzymatically dissociated from the LV midmyocardial layer and stored in normal Tyrode’s solution, as described previously. Cells were placed in a perfusion bath, and all experiments were performed at 37°C. Action potentials were triggered in whole-cell current clamp mode with 2-ms current injections at a cycle length of 2000 ms and recorded with pCLAMP9 software (Axon Instruments). APD at 90% repolarization and STV were calculated from 30 consecutive beats. Both APD and STV were calculated during steady state or just before occurrence of the first early after depolarization.

Experiments were performed in normal Tyrode’s solution containing the following (in mmol/L): 137 NaCl, 5.4 KCl, 0.5 MgCl2, 1.8 CaCl2, 11.8 HEPES, and 10 glucose, at pH 7.4. Pipettes had a resistance of 2–3 MΩ when filled with pipette solution, containing the following (in mmol/L): 130 KCl, 10 NaCl, 10 HEPES, 5 MgATP, and 0.5 MgCl2, at pH 7.2.

Cells were superfused with normal Tyrode’s solution, and after baseline recording dofetilide (1 µM) was added to provoke EADs. After induction of at least 3 EADs by dofetilide (1 µM), antidote was added (see Figure 4A): CSA (1 mg/mL, 4,5 Neoral), or CSA (1 mg/mL, Sigma6).

Protocol E: Intracellular calcium measurements during dofetilide treatment

In isolated CAVB dog cardiomyocytes, calcium transients were determined at baseline and upon superfusion with dofetilide (see Figures 5A and 5B), using a Zeiss Axiovert 100M inverted microscope with a ×40/1.3 oil immersion objective and a Zeiss LSM 510 confocal laser point-scanning system (Zeiss GmbH). Fluo 3 and di-8-ANEPPS were excited using the 488-nm line of a 25-mW argon laser. To detect [Ca2+]i transients, cells were scanned along the longitudinal axis, orthogonal to the Z lines, avoiding scanning through nuclei.

Statistical analysis

The following tests were performed:

- Unpaired t-test: Figures 1B, 1C, 3C, 5C, 5D, and 5E
- Fisher exact test: Figure 4D
- ANOVA followed by Holm-Sidak post hoc test: Figures 1E, 3A, 3B, 3D, and 5G
- Repeated measures ANOVA followed by Holm-Sidak post hoc test: Figures 2C, 2D, 4B, and 4C. In Figure 2C, the comparison in the post hoc test was against the dofetilide group.

References


Online Supplemental Figure 1  APD and STV of individual cells. Every dot represents the APD (left) or STV (right) of a single cell obtained from an individual dog (color code), during baseline measurement, during dofetilide infusion, and after treatment with a subsequent antidote: W-7 in shades of green, KN 93 in brown–red–pink, and cyclosporin A in shades of blue. Dofetilide APD and STV were determined right before the first EADs. Note that in one KN-93 cell and all cells treated with cyclosporin A, APD and STV could not be obtained due to the persistence of EADs.