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Arrhythmogenic right ventricular cardiomyopathy in Boxer dogs is associated with calstabin2 deficiency

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Abstract

Objective—To examine the presence and effect of calstabin2-deficiency in Boxer dogs with arrhythmogenic right ventricular cardiomyopathy (ARVC).

Animals—Thirteen Boxer dogs with ARVC.

Materials and methods—Tissue samples were collected for histopathology, oligonucleotide microarray, PCR, immunoelectrophoresis, ryanodine channel immunoprecipitation and single-channel recordings, and calstabin2 DNA sequencing.

Results—In cardiomyopathic Boxer dogs, myocardial calstabin2 mRNA and protein were significantly decreased as compared to healthy control dogs (calstabin2 protein normalized to tetrameric cardiac ryanodine receptor (RyR2) complex: affected, 0.51 ± 0.04 ; control, 3.81 ± 0.22 ; $P < 0.0001$). Calstabin2 deficiency in diseased dog hearts was associated with a significantly increased open probability of single RyR2 channels indicating intracellular Ca^{2+} leak. PCR-based sequencing of the promoter, exonic and splice site regions of the canine calstabin2 gene did not identify any causative mutations.

Conclusions—Calstabin2 deficiency is a potential mechanism of Ca^{2+} leak-induced ventricular arrhythmias and heart disease in Boxer dogs with ARVC.

Keywords

Ryanodine receptor; Calcium; Dilated cardiomyopathy

Introduction

Sudden cardiac death due to ventricular arrhythmias has been linked to mutations in the cardiac ryanodine receptor (RyR2), the principal intracellular Ca^{2+} release channel of the heart, in

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Disclosures

ARM is a consultant for ARMGO Pharma Inc, a startup company focused on developing novel cardiovascular therapeutics targeting the ryanodine receptor.

humans with arrhythmogenic right ventricular cardiomyopathy (ARVC)^{1–3} and catecholaminergic polymorphic ventricular tachycardia.^{4,5} Boxer dogs exhibit a naturally occurring form ARVC that has clinical and pathologic similarities to ARVC human mutation carriers.⁶ In particular, affected dogs have a high incidence of ventricular arrhythmias with predominantly left bundle branch block morphology that originate from the right ventricle of affected dogs⁷ and are inherited in an autosomal dominant fashion.⁸ Affected dogs are at high risk for sudden cardiac death. Right ventricular myocardium, and frequently the left ventricular and interventricular septal myocardium, is characterized by fatty and fibrofatty replacement, myocyte vacuolization, myocarditis, and necrosis. Clinical signs in affected dogs commonly include syncope, although many individuals are asymptomatic and diagnosed only following fortuitous discovery of arrhythmia during routine examination.

RyR2 controls release of Ca^{2+} sarcoplasmic reticulum stores, which is required for cardiac excitation-contraction coupling. RyR2 channel function is partly modulated by calstabin2, also known as FKBP12.6. Each RyR2 monomer binds 1 calstabin2 subunit, and therefore four calstabin2 molecules are bound to the homotetrameric RyR2 channel. Calstabin2 reportedly stabilizes RyR2 in a closed state and prevents diastolic sarcoplasmic reticulum Ca^{2+} leak, triggered ventricular tachyarrhythmias, and abnormal EC coupling,⁹ although these findings are controversial.^{10–13}

Based on the similarities between canine and human ARVC phenotypes, and that canine RyR2 function relies on a very high calstabin2 binding affinity,¹⁴ we sought to determine whether calstabin2 and its effect on RyR2 function is associated with ARVC in Boxer dogs.

Materials and methods

Animal population

Clinical diagnosis of canine ARVC was made in 13 Boxer dogs based on the presence of >1000 ventricular premature complexes with left bundle branch block morphology on 24-h ambulatory monitoring, and when present, syncope or sudden cardiac death.^{7,15} A three-channel 24 h ambulatory ECG recording system^f was used as previously described.¹⁶ Descriptive data regarding number, morphology, and complexity of premature ventricular beats (PVC) were recorded. Tissues samples from these 13 Boxers were originally collected by two different authors (MAO, KMM) for various purposes (i.e., histopathology, microarray analysis, DNA sequencing, and immunoblotting), and the number of samples used for each subsequent analysis was based on the handling, preparation, and storage of tissues during post-mortem examination. Animal handling was in accordance with the University of Pennsylvania and Columbia University Institutional Animal Care and Use Committees.

Histopathology

Postmortem histopathology on formalin-fixed tissue blocks of ventricular tissue was performed on four Boxer dogs to confirm fatty and fibrofatty infiltration of the ventricular myocardium, which is consistent with canine ARVC.⁶ Full-thickness samples from the right ventricle (RV), left ventricle (LV), and interventricular septum were obtained postmortem. Sections of the left ventricular free wall (1 cm³) were snap-frozen in liquid nitrogen immediately following euthanasia, and stored at –70 °C until processed. Sections of LV, RV, and septum from Boxer dogs were fixed in 10% neutral-buffered formalin for histological examination using hematoxylin and eosin stain.

^fLifecard CF, Spacelabs Heathcare, Issaquah, WA.

Oligonucleotide microarray analysis

Full-thickness samples from the mid-anterior LV of four Boxer dogs with canine ARVC, three Doberman pinscher dogs with dilated cardiomyopathy, three Beagle dogs with experimental heart failure secondary to rapid ventricular pacing, and three healthy purpose-bred mongrel dogs were procured within 15 min after euthanasia and snap frozen in liquid nitrogen. Boxer and Doberman pinscher dogs were electively euthanized at the request of their owners due to progressive heart disease. Diagnosis of dilated cardiomyopathy in Doberman pinscher dogs was made based on presence of myocardial systolic dysfunction, ventricular arrhythmias, and radiographic evidence of congestive heart failure.¹⁷ Dogs with pacing-induced heart failure had undergone between 50 and 80 days of rapid ventricular pacing (180–240 bpm) and exhibited clinical and radiographic evidence of congestive heart failure and echocardiographic evidence of systolic dysfunction. Myocardial function was determined using transthoracic echocardiography^g and calculation of left ventricular fractional shortening. Control dogs included adult mongrel dogs free of cardiac disease.

Messenger RNA levels of calstabin2 were determined using a canine-specific oligonucleotide array as previously described.¹⁸ Briefly, total RNA was isolated and assessed for integrity and quality from LV samples from each of the four patient groups. A single-color canine oligonucleotide microarray^h was used to determine the relative expression of 23,851 canine transcripts. Individual oligonucleotide arrays were performed using samples from each of the four patient groups. Differential expression of calstabin2 between groups was determined by comparing intensities of probe signals using a two-tailed Student's *t*-test (significance designated at values of $P < 0.05$ with Benjamini–Hochberg false discovery correction). Results were reported as the relative fold-change in calstabin2 expression as compared to the control group. Thus, a negative fold-change indicated reduced expression compared to the control group. Differential gene expression for calstabin2 was confirmed by use of real-time quantitative PCR as previously described.¹⁸ Briefly, 1-step RT-qPCRⁱ was performed. Reactions (20 μ L) contained 20 ng of template RNA, 1 $\times$ commercial mix,^j 0.5 U of reverse transcriptase^k/ μ L, 0.5 U of RNase inhibitor/ μ L, and forward and reverse gene-specific primers (concentration of 0.1 μ M each). Relative quantification of gene expression was performed by the $\Delta\Delta$ Ct method with expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as an endogenous-control sample to adjust expression within each sample. The primer pair [F-AGGGACTTGAGCCAGTTACCTTT, R-TGTAAGTCAGCAGCCAACAGAATT] was selected by use of commercially available software^l and synthesized by use of a nucleic acid synthesis and purification system^m in accordance with manufacturer's specifications. All reactions were performed in triplicate. Reactions that did not contain template RNA were included as negative-control samples.

Western blot analysis and immunoprecipitation

Cardiac homogenates were prepared by homogenizing approximately 1.0 g of LV tissue from four Boxer dogs and three Beagle control dogs in 2 ml of homogenization buffer (10 mM Tris-maleate pH 7.4, 0.9% NaCl, 5.0 mM NaF, 1.0 mM Na₃VO₄, and protease inhibitor mixⁿ). Samples were centrifuged at 4000 $\times$ g for 15 min and the supernatant was centrifuged at 12,000 $\times$ g for 15 min. The protein concentration of these tissue homogenates was determined by

^gVivid 7, GE Medical Systems, Waukesha, WI.

^hCanine GeneChip 1.0, Affymetrix, Santa Clara, CA.

ⁱSybrGreen RT-qPCR, Applied Biosystems, Foster City, CA.

^jSybrGreen master mix, Applied Biosystems, Foster City, CA.

^kMultiScribe reverse transcriptase, Applied Biosystems, Foster City, CA.

^lPrimer Express software, Applied Biosystems, Foster City, CA.

^m3948 nucleic acid synthesis and purification system, Applied Biosystems, Foster City, CA.

ⁿComplete Mini, Cat# 04693116001, Roche Diagnostics Corp., Indianapolis, IN.

Bradford and aliquots were stored at -80°C . RyR2 was immunoprecipitated by incubating 250 μg of tissue homogenate with anti-RyR antibody (2 μl 5029Ab) in 0.5 ml of a modified RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 0.9% NaCl, 5.0 mM NaF, 1.0 mM Na_3VO_4 , 1.0% Triton-X100, and protease inhibitor mix for 1 h at 4°C . The samples were subsequently incubated with protein A sepharose beads^o at 4°C for 1 h, after which, the beads were washed three times with RIPA buffer. Lysates (25 μg) were separated using 15% PAGE and immunoblots were probed with anti-calstabin antibody to determine calstabin1 and calstabin2 expression. Cardiac microsomes (20 μg) were prepared to determine RyR2 and calnexin protein expression. Proteins were transferred to nitrocellulose membranes and immunoblots were developed using the following antibodies: anti-calstabin (1:2000),¹⁹ anti-RyR (1:5000 in 5% milk TBS-T),²⁰ anti-phospho-RyR2-pSer²⁸⁰⁸ (1:10,000),²¹ or anti-calnexin (1:5000).²¹ Relative amounts of calstabin2, RyR2, and calnexin were calculated using densitometry of membranes.

RyR2 single-channel recordings

To determine whether calstabin2 deficiency in Boxer dogs with ARVC may affect RyR2 function, we studied single-channel function in lipid bilayers. To prepare vesicles for single channel recordings, LV homogenates from two Boxer dogs and two control dogs were centrifuged at $50,000 \times g$ for 30 min and the pellets were resuspended in homogenization buffer containing 300 mM sucrose. Vesicles containing native RyR2 were fused to planar lipid bilayers in 100- μm holes in polystyrene cups separating two chambers. The *trans* chamber (1.0 ml), representing the intra-SR compartment, was connected to the head stage input of a bilayer voltage-clamp amplifier.^p The *cis* chamber (1.0 ml), representing the cytoplasmic compartment, was held at virtual ground. Symmetrical solutions included the following: *trans*, 250 mM Hepes, 53 mM $\text{Ca}(\text{OH})_2$, pH 7.35; *cis*, 250 mM Hepes, 125 mM Tris, 1.0 mM EGTA, 0.5 mM CaCl_2 , pH 7.35. At the conclusion of each experiment, 5 μM ryanodine or 20 μM ruthenium red was applied to confirm RyR2 channel identity.

DNA sequencing

DNA samples from 10 Boxer dogs with canine ARVC were evaluated. Boxer DNA samples were compared to two unaffected Labrador retriever dogs as well as the published canine (Boxer dog) genome sequence. Genomic DNA samples were prepared from whole blood samples as previously described.²² Briefly, cells were osmotically lysed in $2 \times$ sucrose-Tris- NH_4Cl buffer and nuclei were pelleted by centrifugation at $800 \times g$ for 20 min at 4°C . Pellets were resuspended in saline-EDTA with 1% SDS and 50 $\mu\text{g}/\text{ml}$ proteinase K, and incubated overnight at 56°C . The samples were subjected to two successive phenol:chloroform: isoamyl (25:24:1, pH 8) and one chloroform extraction. Finally, the DNA was ethanol precipitated, washed with 75% ethanol, and resuspended in 250 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). The canine promoter sequence was estimated using Gene2Promoter.^q The polymerase chain reaction amplification primers were designed for the promoter and exon 1 as well as the other three calstabin2 exons using Primer3 software and the canine nucleotide sequence information for *calstabin2* published on the Ensemble database (ENSCAFP00000005884), which accounts for the entirety of the human and canine calstabin2 protein²³ (Table 1). Standard PCR amplifications were carried out using NH_4SO_4 amplification buffer, 0.1 units/ μl reaction volume Taq DNA polymerase, 2.5 mM MgCl_2 , 12.5 μM of each dNTP, 2.5 mM of each PCR amplification primer and 100 ng of template DNA. Samples were denatured for 5 min at 94°C followed by 40 cycles of 94°C for 20 s; 58°C for 30 s, 72°C for 30 s; and finally 72°C for 7 min. The annealing temperature was optimized to accommodate

^oAmersham Pharmacia Biotech, Piscataway, NJ.

^pWarner Instruments, Hamden, CT.

^qGene2Promoter, Ann Arbor, Michigan.

the respective primer requirement. Residual amplification primers and dNTPs were removed from the PCR product using ExoSapIt enzymatic treatment.[†] Amplicons were then subjected to nucleotide sequence determination and analyzed on an ABI Prism 377 Sequencer[§] using a forward and reverse primer for each reaction for every sample.

The sequences were compared for nucleotide sequence changes between affected dogs, the published normal canine sequence (derived from a Boxer dog) and the controls. Base pair changes were considered to be causative for canine ARVC if they met the following criteria: were present in all of the affected dogs, changed a conserved amino acid, and changed the amino acid to one of a different polarity, acid/base status or structure.

Statistical analysis

Analysis between the experimental groups were performed by using unpaired Student's *t*-tests or one-way ANOVA and Tukey multiple comparison tests when comparing multiple groups. $P < 0.05$ was considered significant.

Results

Boxer dog electrocardiograms

Dogs with ARVC demonstrated isolated PVCs of LBBB morphology as well as couplets, triplets, ventricular tachycardia, and R on T phenomenon (Fig. 1). The mean number of PVCs over a 24 h period was 29,782 (range; 1482–91,000). Twelve of 13 dogs (92.3%) demonstrated multiform PVCs and episodes of ventricular tachycardia.

Histopathology

Boxer dogs possessed histopathologic abnormalities consistent with previous reports of canine ARVC.⁶ Specifically, RV, LV, and interventricular septal tissue displayed myocyte loss, vacuolization, and infiltration with adipose tissue (Fig. 2), consistent with a generalized ventricular cardiomyopathy.

Oligonucleotide microarray analysis

Transcriptional activity of calstabin2 (Affymetrix ID, 1588953) was significantly different between the four groups of dogs ($P < 0.0002$) (Fig. 3). Calstabin2 mRNA in Boxer dog hearts with ARVC was significantly lower than healthy controls and Doberman pinschers with dilated cardiomyopathy (fold change in Boxer dogs: -13.3 vs. control, $P < 0.001$; -7.0 vs. Doberman pinschers, $P < 0.05$). Microarray data for calstabin 2 were validated using real time RT-qPCR, which indicated a -12.5 fold-change in Boxer dogs vs. controls ($P < 0.05$). Microarray analysis found no significant difference in SERCA2, SERCA1, or RYR2 expression between groups (data not shown). MIAME-compliant data from the oligonucleotide microarray analysis was deposited in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) for public access (Accession#, GSE11015).

Calstabin2 deficiency in myocardial tissue and in the RyR2 macromolecular complex

Immunoprecipitation of RyR2 followed by immunoblotting from LV homogenates revealed significantly decreased calstabin2 levels in the RyR2 channel complex of affected Boxer dogs with ARVC when compared to control dogs (Fig. 4A). Relative amounts of calstabin2 and RyR2 were calculated using densitometry of gels and indicated significantly reduced calstabin2 in the RyR2 complex in the hearts of affected Boxer dogs versus control (calstabin2 per RyR2

[†]Amersham Biosciences, Piscataway, NJ.

[§]Applied Biosystems, Foster City, CA.

complex: Boxer dogs, 0.51 ± 0.04 vs. control, 3.81 ± 0.22 ; $P < 0.0001$; Fig. 4B). PKA phosphorylation of RyR2-Ser²⁸⁰⁸ was not significantly different between Boxer dogs and controls (Fig. 4A). In whole cardiac LV tissue homogenates (as opposed to isolates of RYR2 protein) immunoblotting of calstabin detected both calstabin1 and calstabin2 isoforms. Calstabin1 binds preferentially to RYR1, while calstabin2 binds to RYR2, which is the dominant form of RYR found in cardiac tissue.^{14,24} While calstabin1 concentrations were unchanged, calstabin2 concentrations showed a significant reduction of protein expression consistent with reduced mRNA expression shown earlier (Fig. 4C). A reduced RyR2 concentration has been reported in Boxer ARVC dogs in one study,²⁵ however in our study both RyR2 protein level and RyR2 normalized to an internal control, calnexin, did not reach statistical significance (Fig. 4D).

Calstabin2 depletion results in RyR2 dysfunction

RyR2 channels from Boxer ARVC hearts showed significantly increased activity and open probability (P_o) at 150 nM *cis* Ca²⁺ when the channel is supposed to be mainly closed (Fig. 5). A significantly increased P_o and reduced closed time (T_c) indicate a gain-of-function defect in Boxer ARVC RyR2 channels.

DNA sequencing

No differences were observed within the promoter, the four exonic, or splice site regions of the calstabin gene between the affected Boxer dogs and the controls or published Boxer sequence using the transcript we selected. Conservation of the nucleotides of the four canine calstabin2 exonic coding regions ranged from 91 to 98% as compared to human calstabin2 gene.

Discussion

Our study identifies for the first time a molecular mechanism involving RyR2 dysfunction due to depressed calstabin2 expression and intracellular Ca²⁺ leak in Boxer dog ARVC. In our study we report markedly decreased myocardial calstabin2 expression, resulting in calstabin2 depletion in the RyR2 complex, as well as calstabin2 depletion within homogenates of whole cardiac LV tissue. Calstabin2 depletion from RYR2 has been previously demonstrated in both human²⁶ and animal¹¹ models of HF.²⁷ A study in dogs with pacing-induced HF revealed significantly decreased calstabin2 protein in the RyR2 complex as compared to control.¹¹ PKA hyperphosphorylation of RyR2, a defect that is variably associated with the heart failure phenotype, chronic catecholamine stimulation, and depletion of calstabin2 from RyR2,^{26,28} was not found in Boxer dogs with ARVC, implicating reduced expression and decreased calstabin2 protein as likely mechanism of calstabin2 deficiency in the RYR2 channel complex.

A previous study indicated that RyR2 message and protein was decreased in both the RV and LV in Boxer dogs with ARVC, with the greatest reductions occurring in the RV.²⁵ In our study, by immunoprecipitating the RyR2 macromolecular complex, we were able to standardize the amount of RyR2 protein used for immunoblotting and directly compare calstabin2 in the RyR2 complex between study groups. We found calstabin2 depletion in the LV of Boxer dog ARVC hearts, and this finding along with the presence of fatty infiltration of the LV, suggests that further comparative studies using RV and interventricular tissue from affected dogs and controls should be performed.²⁹

Previous studies in Boxer dogs were not able to link ARVC to either the RyR2 gene²⁵ or five different desmosomal genes, including plakophilin-2, plakoglobin, desmoplakin, desmoglein-2, and desmocollin.³⁰ In our study, direct sequencing of DNA samples from 10 affected Boxers with ARVC did not identify a causative mutation in the estimated promoter,

exonic, or splice site regions of calstabin2. The mechanisms underlying the decreased calstabin2 mRNA expression in Boxer dogs with ARVC requires further investigation and may be associated with decreased mRNA stability and/or abnormalities of calstabin2 transcription.

Our study has several limitations. One criticism may be the use of non-Boxer dogs as controls and our findings should be corroborated by additional studies using well-defined control groups of healthy Boxers. In this study, we chose to use non-Boxer controls to avoid the inadvertent inclusion of individuals affected by subclinical (concealed) cardiomyopathy as a result of the high prevalence of ARVC in Boxer dogs¹⁵ and felt that significant breed differences involving important cardiac proteins were unlikely. Likewise, we compared our Boxer dog DNA samples to two Labrador retriever dogs since the published canine genome was developed from an adult Boxer dog that may not have been evaluated for ARVC.³¹ Another limitation involves the examination of RYR2 function from only LV samples of Boxer dogs, and further studies utilizing RYR2 isolated from the interventricular septum and RV of both control and affected animals are warranted. Our tissue samples were those of “convenience”, that is, gathered from a tissue bank that had been previously collected by the investigators (MAO, KMM). Due to the desire to compare different forms of canine cardiomyopathy, and the unavailability of RV tissue samples from control and Doberman pinschers with DCM, we chose to utilize LV samples from all groups. A third important limitation involves uncertainty if the calstabin2 deficiency we report is a primary or secondary abnormality. Boxer dogs demonstrated decreased calstabin2 expression compared to another form of naturally-occurring DCM in Dobermans, however expression in dogs undergoing rapid ventricular pacing was similar to that found in Boxers. Interestingly, all forms of cardiomyopathy evaluated in this study demonstrated decreased calstabin2 expression as compared to control, highlighting the possibility that decreased expression could be a secondary change in response to the heart failure phenotype; however in another breed of dog (Great Dane), which is also commonly afflicted with cardiomyopathy, calstabin2 expression as measured by a second generation oligonucleotide microarray is upregulated (unpublished data) and these apparent differences warrant further study. Even if calstabin2 deficiency is a secondary abnormality, restoration of calstabin2-RYR2 stoichiometry may be an attractive therapeutic target to help reduce incidence of arrhythmias.³⁰ Finally, Xiao et al. recently reported the absence of inducible ventricular arrhythmias in calstabin2-null mice even when stimulated with epinephrine or caffeine,¹⁰ and further studies that specifically investigate the effect of RYR2 dysfunction on production of arrhythmias in Boxer dogs are needed.

Our findings of calstabin2 depletion in the RyR2 complex in the hearts of Boxer dogs with arrhythmogenic cardiomyopathy indicate a specific molecular mechanism with direct pathophysiological implications for the canine disease phenotype. Our results, together with previous studies, indicate that Boxer dogs with calstabin2 deficiency may represent a potentially important abnormality in Boxer ARVC.

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Figure 1.

Affected Boxer dogs manifest ventricular arrhythmias with left-bundle branch block morphology. Representative 24-h ambulatory electrocardiographic recordings from Boxer dogs with ARVC showing (A) ventricular bigeminy, (B) multiform premature ventricular complexes and (C) ventricular tachycardia with a left bundle branch block morphology. 25 mm/s, 0.5 cm/mV.

Figure 2.

Affected Boxer dogs display histological abnormalities. Representative histopathological examination of myocardium from the (A) right ventricle, (B) interventricular septum, and (C) left ventricle from Boxer dogs with arrhythmogenic cardiomyopathy reveal multifocal myocyte vacuolization, myocyte loss, and fatty infiltration. A, 5 × magnification, bar = 200 μm; B and C, 10× magnification, bar = 100 μm; hematoxylin and eosin.

Figure 3.

Affected Boxer dogs display decreased transcriptional activity of calstabin2 as compared to control, Doberman pinschers with dilated cardiomyopathy, and dogs undergoing rapid ventricular pacing. Bar graph displaying relative transcriptional activity of calstabin2 in control, Doberman pinschers with dilated cardiomyopathy (DCM), dogs undergoing rapid ventricular pacing (Pacing), and Boxer dogs with arrhythmogenic right ventricular cardiomyopathy (Boxer) as measured by oligonucleotide microarray. *, $P < 0.05$ vs. control; †, $P < 0.01$ vs control; ‡, $P < 0.001$ vs. control; §, $P < 0.05$ vs. DCM.

Figure 4.

Boxer dogs with arrhythmogenic cardiomyopathy show decreased calstabin2 levels in the RyR2 channel complex. Cardiac lysates and SR microsomes were prepared from control animals and Boxer dogs with arrhythmogenic right ventricular cardiomyopathy (ARVC) as described in Section 2. (A) RyR2 was immunoprecipitated followed by immunoblotting as indicated. Calstabin2 is apparently decreased in the RyR2 complex while PKA phosphorylation at Ser²⁸⁰⁸ is unchanged. (B) Bar graph showing that calstabin2 normalized to cardiac RyR2 is significantly decreased in the channel complexes of Boxer dogs. *, $P < 0.0001$. (C) Immunoblot from whole cardiac tissue lysates (200 μ g) separated by 15% PAGE developed with anti-calstabin antibody. Both calstabin1 (lower band corresponding to 12 kDa, indicated by dotted

line and arrow) and calstabin2 (upper band corresponding to 12.6 kDa, indicated by solid line and arrow) are detected by the antibody in control dogs. Boxer dogs show reduced calstabin2 expression. Calstabin1 is present in cardiac tissues but has a much lower affinity for RYR2 than calstabin2. (D) RyR2 and calnexin protein levels from SR microsomes of control and Boxer dogs. (E) Quantification of RyR2 and calnexin is displayed in the left and middle panel, and RyR2 normalized to calnexin protein levels is shown in the right panel. No significant changes were detected (*P* values as indicated).

Figure 5.

Single RyR2 channels from the hearts of cardiomyopathic Boxer dogs show a defective function. (A) Representative current traces of RyR2 channels from two control and two Boxer dogs with arrhythmogenic right ventricular cardiomyopathy (ARVC) as indicated. C, indicates closed state, full open level is 4 pA. Upper traces represent 5 s, lower traces 500 ms. Current amplitude histograms confirm partial channel openings, indicating subconductance states. (B) Summary bar graphs of open probability (Po), and mean open (To) and closed (Tc) times document significant increase in Po and a parallel significant decrease in Tc in Boxer dog channels versus control ($P < 0.001$ each). Bilayer conditions approximate the resting phase of the heart (diastole; free *cis* $[Ca^{2+}]$ 150 nM). Experiments are representative of $n = 12$ from two control dog hearts and $n = 13$ from two boxer ARVC dog hearts.

Table 1

Polymerase chain reaction amplification primers use to evaluate the promoter and exons 1–4 of the canine calstabin2 gene

Exon	Forward primer	Reverse primer
Promoter and exon 1	GGCAGTGAGCGCATCTAT	CTCCGACAGTGCAGTCGT
Exon 2	GTCCCAGGTGACTTTTCCA	CCTGATCCAGGGAGCAAATA
Exon 3	CCACAGCTCTTCTCCATCCT	CAGCGGTCTGTGATAGGTCA
Exon 4	AAGCTGGGTCCTCTTCTCC	CGTTAGCGCACTGATCAAGA