The multifunctional role of calcium in the heart: a tempting target

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The multifunctional role of calcium in the heart: a tempting target

De multifunctionele rol van calcium in het hart: een verleidelijk doelwit

(met een samenvatting in het Nederlands)

Proefschrift

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Preface

V.J.A. Bourgonje

Abbreviations:
AP: Action potential
CaMKII: Calcium/calmodulin-dependent protein kinase II
LTCC: L-type calcium channel
NCX: Sodium/calcium exchanger
RyR: Ryanodine receptor
SERCA: Sarcoplasmic reticulum calcium ATPase
SR: Sarcoplasmic reticulum

Preface

For all its complexity, the heart has a simple task: to pump blood efficiently.
In order to achieve this, the following prerequisites are necessary:

1) The heart must be *excited* in a regular and ordered manner.
2) The heart must *contract* with sufficient force.
3) The *structural dimensions* must be balanced in order to maintain optimal wall strain.

Remarkably, calcium plays a pivotal role in all of these demands. As a charged ion moving across the cellular membrane it is involved in *excitation*, both in sino-atrial cells where it is responsible for the upstroke, as well as in ventricular myocytes where its charge is one of the factors responsible for the plateau phase of the action potential.
During the plateau phase it is also involved in *contraction*, as calcium binds to troponin and thereby allows the myofilaments to exert force. In fact, the sudden increase in intracellular calcium concentrations $[Ca^{2+}]_i$ during every heart beat is the key element in excitation-contraction coupling. Concerning *structural dimensions*, calcium is again of prime importance. Calcium-sensitive signaling proteins, like calcineurin and CaMKII, translate changes in calcium concentration to altered expression patterns, potentially leading to cardiac hypertrophy and remodeling. Given the central role of calcium in the heart it comes as no surprise that disturbances in calcium handling can have catastrophic effects like ventricular arrhythmias, diastolic failure, and dilated cardiomyopathy. Obviously, this makes it a highly attractive therapeutic target as well. However, due to its multi-functionality, preventing unwanted side effects on top of the desired therapeutic effects will be a challenge. Whoever is able to unravel this dilemma, will open up a whole new field of therapeutic possibilities.

Calcium in excitation and contraction
As a charged ion moving across the cellular membrane, calcium is involved in excitation. Since calcium binds to troponin and thereby activates the myofilaments, it is also involved in contraction.
In the ventricular cardiomyocyte, excitation starts when neighbouring cells transfer positive ions through gap junctions. Once the membrane potential starts to rise, sodium channels open and a subsequent influx of positive sodium ions leads to rapid depolarization. Upon this depolarization, voltage sensitive LTCCs open, leading to an inward calcium flow. In a process called calcium-induced-calcium-release, this triggers the opening of RyR channels on the SR that in turn give rise to a massive increase in the intracellular calcium concentration. With these higher concentrations, calcium can bind to troponin, and contraction can occur. The time to enable this binding and cellular shortening, is provided by the the plateau phase of the AP for which calcium is mainly responsible. In this way it is the change in intracellular calcium concentration that couples excitation to contraction.
Subsequently, contraction turns into relaxation once the calcium concentration starts to fall because calcium is pumped back into the SR via SERCA and a smaller amount of calcium is removed out of the cell via the NCX. This last part leads to a net depolarizing current, as 3 sodium ions are transported in for every calcium ion out. However, during that phase of the physiological action, repolarizing potassium currents dominate, in effect neutralizing this inward current and repolarizing the cell to the resting membrane potential.
Theoretically, it might be possible to discern the role of calcium in excitation from the one in contraction, but in practice they are so closely intertwined that it is almost impossible to influence one without the other. For more in depth information concerning the ion currents

involved in action potential formation and for calcium handling, I kindly refer to chapter 2 after this preface.

Calcium in arrhythmias and contraction

Pro-arrhythmic effects of calcium are based on the same mechanisms present in normal excitation: calcium currents are positively charged inward currents. Therefore, untimely calcium currents can cause triggered activity, either because the LTCC re-activates at the end of the action potential, or because calcium is uncontrollably released from intracellular calcium stores like the SR, leading to a depolarizing current via the NCX [1]. These latter changes to the AP are referred to as afterdepolarizations, and are seen in pathophysiological conditions, in which excitation contraction is altered (ventricular remodeling).
Not surprisingly, to temper these pro-arrhythmic currents, pharmaceutical interventions have been developed. In fact, class IV of the Vaughan-Williams classification of anti-arrhythmic drugs consists entirely of calcium channel blocking agents [2]. Although effective, the usefulness of these drugs is hampered by their negative inotropic effects, which is understandable given the role of calcium in cardiac contraction [3].
Inhibiting calcium currents will decrease triggered activity. Conversely, blocking the depolarizing current through NCX can potentially reduce afterdepolarizations as well, but runs the risk of slowing calcium removal and thus impair relaxation. Either way, it is difficult to target the electrical effects of calcium without influencing the inotropic effects as well.

Calcium-dependent signaling and structural remodeling

Next to excitation and contraction, calcium is also involved in intracellular signaling. It acts as a second messenger by activating the signaling proteins CaMKII and calcineurin. Both are involved in changes in gene expression related to pathological hypertrophy, characterized by dilatation, decreased fractional shortening, and pro-arrhythmic changes like deposition of fibrosis and reduced intercellular coupling [4, 5]. Under these circumstances arrhythmias might be triggered, but can also result from reduced conduction velocity and increased propensity to generate re-entry circuits[6, 7]. In addition, CaMKII can also directly phosphorylate ion channels and in that way induce afterdepolarizations [8]. Whereas calcineurin seems to be activated by prolonged increased diastolic calcium concentrations, CaMKII has been shown to be activated by increased frequency and longer action potentials. Calcium therefore, does not only link excitation to contraction, but also to long-term structural changes (ventricular remodeling). It is a pivotal molecule indeed.

Calcium and research

Despite its central role in cardiac function and dysfunction, class IV anti-arrhythmics are the only clinical interventions targeting calcium handling directly. β-blockers are much more a-specific. Considering that the only calcium directed intervention is associated with negative hemodynamic effects, this obviously merits further research. The central theme of this thesis hinges on all three afore mentioned (excitation, contraction, signaling) roles of calcium in the heart, and we have tested interventions both to suppress arrhythmias while maintaining contraction, as well as cardiac remodeling. The general hypothesis of this thesis is therefore:

Targeting calcium is an effective strategy both to acutely inhibit arrhythmias while preserving cardiac function, and to prevent chronic pathological cardiac remodeling

Specifically, we have investigated the effects elicited by acute and chronic CaMKII inhibition with respect to anti-arrhythmia and prevention of cardiac remodeling; a field still relatively new and unexplored, but with great potential. Next to that, we have also investigated whether

it is possible to be anti-arrhythmic, while preserving cardiac function. For that we performed a translational study using SEA 0400, a (combined) blocker of the NCX (and LTCC).

The outlay of the thesis follows this duality. First, **Chapter 2** introduces physiological excitation-contraction coupling as well as mechanisms of arrhythmogenesis. In **chapter 3** an overview of pro-arrhythmic ventricular electrical remodeling is given, followed in **chapter 4** by a study on CaMKII inhibition in a canine long-QT model.
In **chapters 5 and 6,** we describe the effects of chronic CaMKII inhibition on pro-arrhythmic and pathological remodeling in a mouse model of pressure overload and a mouse model with calcineurin-induced cardiomyopathy, respectively.
Chapter 7 summarizes the mechanism behind the anti-arrhythmic efficacy, but hemodynamic intolerance, of class IV anti-arrhythmics, using the LTCC blocker verapamil as an example. Consequently, **chapter 8** is about the combined LTCC and NCX blocker SEA-0400, where we were able to show minimal deleterious hemodynamic effects while still providing a complete anti-arrhythmic effect. **Finally, Chapter 9** comprises the general discussion.

Reference List

(1) Antoons G, Willems R, Sipido KR. Alternative strategies in arrhythmia therapy: evaluation of Na/Ca exchange as an anti-arrhythmic target. *Pharmacol Ther* 2012 April;134(1):26-42.

(2) Zipes DP, Camm AJ, Borggrefe M et al. ACC/AHA/ESC 2006 Guidelines for Management of Patients With Ventricular Arrhythmias and the Prevention of Sudden Cardiac Death: a report of the American College of Cardiology/American Heart Association Task Force and the European Society of Cardiology Committee for Practice Guidelines (writing committee to develop Guidelines for Management of Patients With Ventricular Arrhythmias and the Prevention of Sudden Cardiac Death): developed in collaboration with the European Heart Rhythm Association and the Heart Rhythm Society. *Circulation* 2006 September 5;114(10):e385-e484.

(3) Oros A, Houtman MJ, Neco P et al. Robust anti-arrhythmic efficacy of verapamil and flunarizine against dofetilide-induced TdP arrhythmias is based upon a shared and a different mode of action. *Br J Pharmacol* 2010 September;161(1):162-75.

(4) Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol* 2006 August;7(8):589-600.

(5) Anderson ME, Brown JH, Bers DM. CaMKII in myocardial hypertrophy and heart failure. *J Mol Cell Cardiol* 2011 October;51(4):468-73.

(6) Bierhuizen MF, Boulaksil M, van Stuijvenberg L et al. In calcineurin-induced cardiac hypertrophy expression of Nav1.5, Cx40 and Cx43 is reduced by different mechanisms. *J Mol Cell Cardiol* 2008 September;45(3):373-84.

(7) de Jong S, van Veen TA, van Rijen HV, de Bakker JM. Fibrosis and cardiac arrhythmias. *J Cardiovasc Pharmacol* 2011 June;57(6):630-8.

(8) Anderson ME. Multiple downstream proarrhythmic targets for calmodulin kinase II: moving beyond an ion channel-centric focus. *Cardiovasc Res* 2007 March 1;73(4):657-66.

Chapter 2:

Introduction to physiological excitation-contraction, impulse propagation, and arrhythmogenic mechanisms

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Abbreviatons:
AP(D): action potential (duration)
CaMKII: Calcium/calmodulin-dependent protein kinase II
CICR: Calcium-induced calcium release
CV: Conduction velocity
Cx: Connexin
DAD: delayed afterdepolarization
EAD: early afterdepolarization
ERP: effective refractory period
LTCC: L-type calcium channels
NCX: Na^{+}/Ca^{2+} Exchanger
PKA: Protein kinase A
PLB: Phospholamban
RyR: ryanodine receptor
SR: Sarcoplasmic reticulum
SERCA: SR Ca^{2+}-ATPase

Overall, the cardiac rhythm is quite regular but can nonetheless respond adequately to changes in demand by changing its beating rate. Every beat is a complex and highly structured chain of events. If anywhere along this chain malfunctions occur, the result can be an arrhythmia.In this chapter impulse propagation is described, as well as excitation -contraction coupling on the cellular level. Dysfunction in the first can lead to re-entry based arrhythmias, while problems in the latter can be linked to triggered arrhythmias, which will also be described in this chapter.

Impulse Propagation

For the heart to function properly, excitation and contraction of all the myocytes in the heart needs to be coordinated and balanced. Therefore the electrical impulse that initiates excitation moves throughout the heart via a specific route starting in the sinoatrial node. Next the atria are activated, after which the electrical signal travels down through the bundle of His and the bundle branches towards the apex of the heart where it activates the ventricular myocytes from apex to base via the purkinje fibers and anisotropic fiber structure[1]. This leads to a coordinated contraction of both ventricles. For the signal to travel from myocyte to myocyte they need to be coupled, which is facilitated by gap junctions which are composed of connexins. In the heart three types of connexins are present. Cx40 is mainly expressed in the atria and throughout the conduction system. Cx43 is the most abundant Cx isoform in both the atria and ventricles but is also found in the distal conduction pathway. Finally, Cx45 is only found in the atrioventricular node, His bundle, and bundle branches. For a detailed review of cardiac connexins see: Jansen et al.[2]. The connexins are localized in the intercalated discs that are situated at the longitudinal cell-edges where they facilitate the connection between the myocytes. It is this localization which supports conduction in an anisotropic manner; with a faster conduction along the fiber orientation (longitudinal conduction) compared to the conduction perpendicular to that orientation (transverse conduction).

Ions → APD

The action potential is generated via complex interaction of ion channels and membrane voltage, and is generally divided in five phases. They are established through a fine-tuned interaction of sodium, potassium, and calcium currents. The inward Na^+ current (I_{na}) is responsible for the upstroke during phase 0 (figure 1, phase 0). During phase 1 the Na^+ channels inactivate and at the same time I_{to1} and I_{to2} (the transient outward currents) create outward currents of potassium and chloride respectively to form the notch (figure 1, phase 1). Calcium enters the cell through voltage gated calcium channels (LTCC) and is involved in creating the plateau phase together with the delayed rectifier potassium currents I_{ks} and I_{kr} (figure1, phase 2). The plateau phase delays the repolarization of the action potential creating time for contraction and relaxation of the cardiomyocytes before the next action potential is generated. Repolarization occurs when the LTCC closes and the delayed rectifier potassium channels remain open (figure 1, phase 3). Finally, potassium restores the negative membrane potential in phase 4 via the I_{k1} current (figure 1, phase 4).

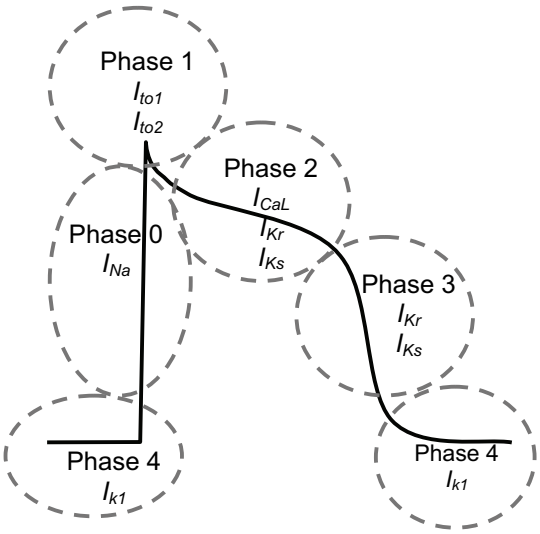

Figure 1. Action potential and ion currents. Phases of the action potential and the responsible ion currents.

Excitation-contraction coupling

Of the ions involved in the activation of the heart, calcium plays a key role in excitation-contraction. Ca^{2+} has effects on the membrane potential during the AP plateau via LTCC. LTCC is activated upon depolarization of the sarcolemma due to an increase in $[Na^+]_i$, but inactivated by local $[Ca^{2+}]_i$ via calmodulin binding on the C-terminus of the channel. The initial calcium influx via the LTCC leads to calcium-induced-calcium-release from the SR which is mediated by the ryanodine receptors. Once RyRs open, this leads to Ca^{2+} extrusion from the SR and hereby cytosolic $[Ca^{2+}]$ increases while also calcium-dependent inactivation of the LTCC is triggered [3]. The RyR is a channel, but also a scaffolding protein that clusters proteins like calmodulin/CaMKII (exerts Ca^{2+} dependent modulation of RyR and LTCC, see below), PKA (which can alter RyR and LTCCgating), and sorcin (which connects RyRs and LTCCs) near the calcium release complex [3]. Subsequently, calcium binds to troponin to facilitate contraction of the sarcomere, the contractile element of the myocyte [3]. Thus, Ca^{2+} links the electrical activation of cardiomyocytes to mechanical contraction, which is called excitation-contraction coupling [3] (see also: figure 2).

During relaxation, the cytosolic $[Ca^{2+}]$ has to decline to allow Ca^{2+} to dissociate from troponin leading to relaxation of the contractile element. This process is facilitated by SERCA transporting Ca^{2+} back into the SR, and NCX on the cellular membrane, which is depicted in figure 2. [3].

The NCX exchanges three Na^+ ions for one Ca^{2+} ion. This exchange can go in both directions and is dependent on Na^+ and Ca^{2+} concentrations as well as the membrane potential. Whether the current is named inward or outward depends on the direction of the Na^+ current. High $[Ca^{2+}]_i$ favors inward $I_{na/ca}$, whereas high $[Na^+]_i$ and positive membrane potential favors outward $I_{na/ca}$ [3].

SERCA is an active calcium pump that can be inhibited via PLB, which in turn is inhibited by PKA dependent phosphorylation. Activators of PKA, like adrenergic stimulation, can therefore play a role in relaxation, as because of higher SERCA activity calcium is removed faster from the cytosol which means that contraction will end quicker. This actually also helps contraction, as the higher SERCA activity means more calcium is stored in the SR,

subsequently rendering more available calcium for CICR, and therefore induced force of contraction.
CaMKII is known to influence this calcium handling on multiple levels (figure 2). It can directly phosphorylate the LTCC and RyR, both resulting in a gain-of-function. It is also able to phoshorylate PLB, which in effect diminishes the inhibitory effect of PLB, leading to more SERCA activity (comparable to the effects of PKA-dependent phosphorylation). Consequently, this results in higher SR calcium load, higher calcium fluxes and increased contraction.

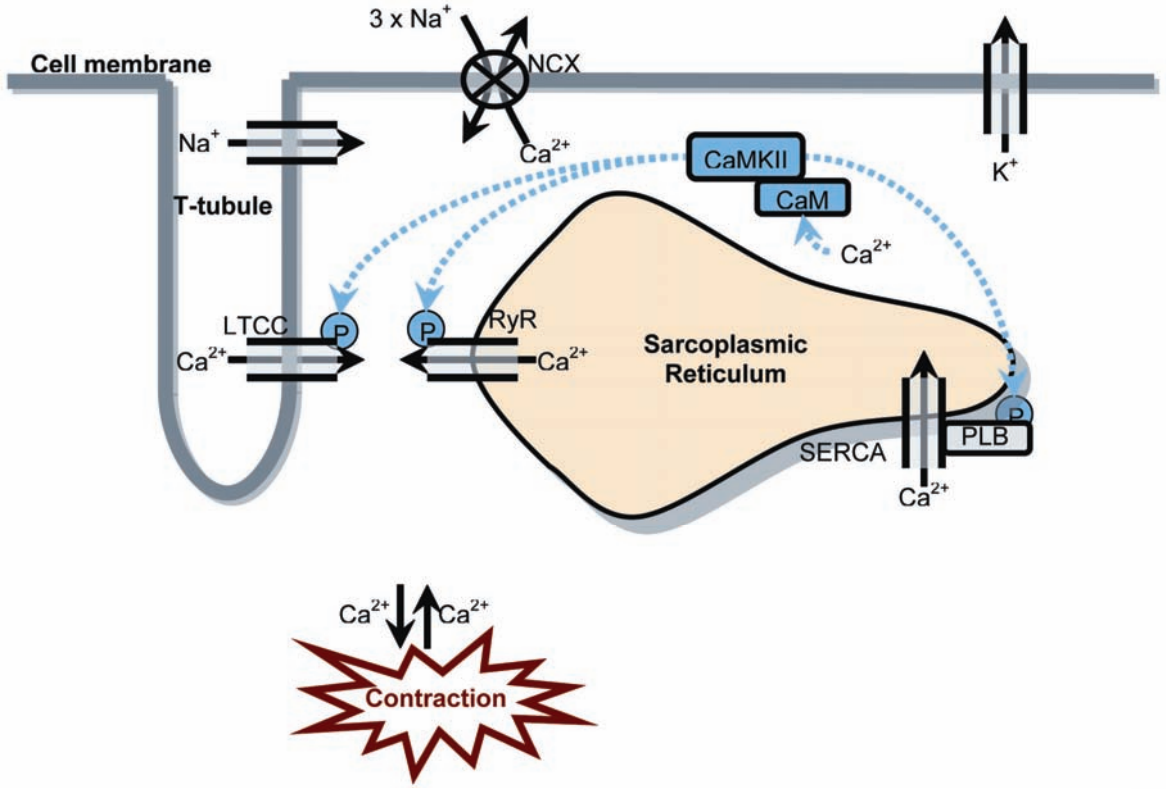

Figure 2. CaMKII interaction with calcium handling. 0; Sodium enters the cell, creating the AP upstroke. 1; Calcium enters via the LTCC facilitating the plateau phase of the AP and initiating CICR, via RyR (2) on the sarcoplasmic reticulum leading to 3; calcium binding to the contractile elements: excitation-contraction coupling. 4; NCX transports calcium form the cell in exchange for sodium leading to relaxation of the contractile elements and the end of the plateau phase. 5; Potassium restores the negative membrane potential.

Re-entry arrhythmias

Pathological remodeling of the highly homogeneous expression pattern of gap junctions may contribute to generation of a pro-arrhythmic substrate that may increase the propensity for re-entry based arrhythmias. In addition, focal uncoupling of the syncytium may favor the occurrence of ectopic activity. The mechanisms that underlie ectopic activity will be discussed further on, and provide potential new targets for anti-arrhythmic interference.
Re-entry occurs when an electrical impulse does not end, but instead reactivates tissue in a circular manner. Re-entry requires a unidirectional block, and success or failure of the reentrant path is dependent on conduction velocity and tissue refractoriness. The stimulus needs to travel slowly enough so the ERP of the tissue has passed. Therefore the path length of the reentrant wave can not exceed the wavelength (product of CV and refractoriness)[4].
Uncoupling of myocytes due to heterogeneous distribution of Cx43, which probably coincides

with sodium current downregulation, leads to dispersed conduction and a decrease in CV, creating an arrhythmogenic substrate [5, 6]. Also, reduced Cx43 expression is correlated to enhanced fibroblast activity, resulting in fibrosis, leading to even more dispersed conduction and further uncoupling of myocytes [5].

Triggered arrhythmias

In heart failure, calcium handling is disturbed, as functional expression of SERCA is reduced and NCX exchange is increased [3]. Moreover, kinetics of RyR opening are also changed, leading to calcium disturbances which can be a substrate for arrhythmias based on triggered activity like early and delayed afterdepolarizations. EADs and DADs are defined as: oscillations that attend (EADs) or follow (DADs) the cardiac action potential and respond on preceding activation for their manifestation. When the amplitude of the depolarization reaches threshold, triggered activity occurs[7].

EADs

EADs are generated in the ventricular myocyte (or Purkinje fibers) during phase two-three of the AP, and can occur during very slow heart rates (bradycardia) with long action potentials[8]. At the end of the prolonged APD, EADs can occur either via a LTCC window current[9], or via calcium leak from the SR (figure 3B). Both situations enhance $[Ca^{2+}]_i$ leading to increased Ca^{2+} removal from the cell via NCX. NCX exchanges 3 Na^+ ions for 1 Ca^{2+} ion leading to a net positive inward current with a potential depolarizing effect leading (under certain conditions) to EADs or DADs (see below) [10]. Furthermore, in a feed forward effect, the net positive inward current produced by NCX can activate LTCC, which leads to increasing $[Ca^{2+}]_i$, thereby having a synergistic effect to the explained functioning of NCX [7]. Whether LTCC or NCX has a dominant role in creating EADs, depends on the disease setting; in heart failure and ischemia reperfusion LTCC is dominant, in a β-adrenergic setting NCX is dominant [7]. Either way, NCX and LTCC are important players in the generation of EADs.

DADs

DADs arise after full repolarization of the myocyte. Ca^{2+} release from the SR is triggered when free $[Ca^{2+}]_{SR}$ reaches a certain threshold leading to RyR opening, see figure 4 [3]. $[Ca^{2+}]_{SR}$ is influenced by total $[Ca^{2+}]_{in}$ and the activity of SERCA. Increased $[Ca^{2+}]_{SR}$ load due to higher SERCA activity, via β-adrenergic stimulation for example, brings the $[Ca^{2+}]_{SR}$ closer to the threshold of SR leak. Furthermore, the threshold is affected by RyR open probability: if the open probability increases the threshold lowers. The open probability of RyR is modulated by $[Ca^{2+}]_{SR}$, $[Ca^{2+}]_{tot}$, APD, and RyR phosphorylation [11, 12]. RyR phosphorylation is executed by CaMKII, which increases the open probability of RyR [11]. Opening of multiple RyRs creates Ca^{2+} sparks, which can lead to DADs. If the DADs reach AP threshold a new AP arises. In heart failure $[Ca^{2+}]_{SR}$ and threshold are both lowered figure 4), yet, threshold is affected more than $[Ca^{2+}]_{SR}$ leading to a higher occurrence of triggered arrhythmias in these patients [13]. The role of NCX in DADs may be comparable to its role in EADs (figure 3B), as described above.

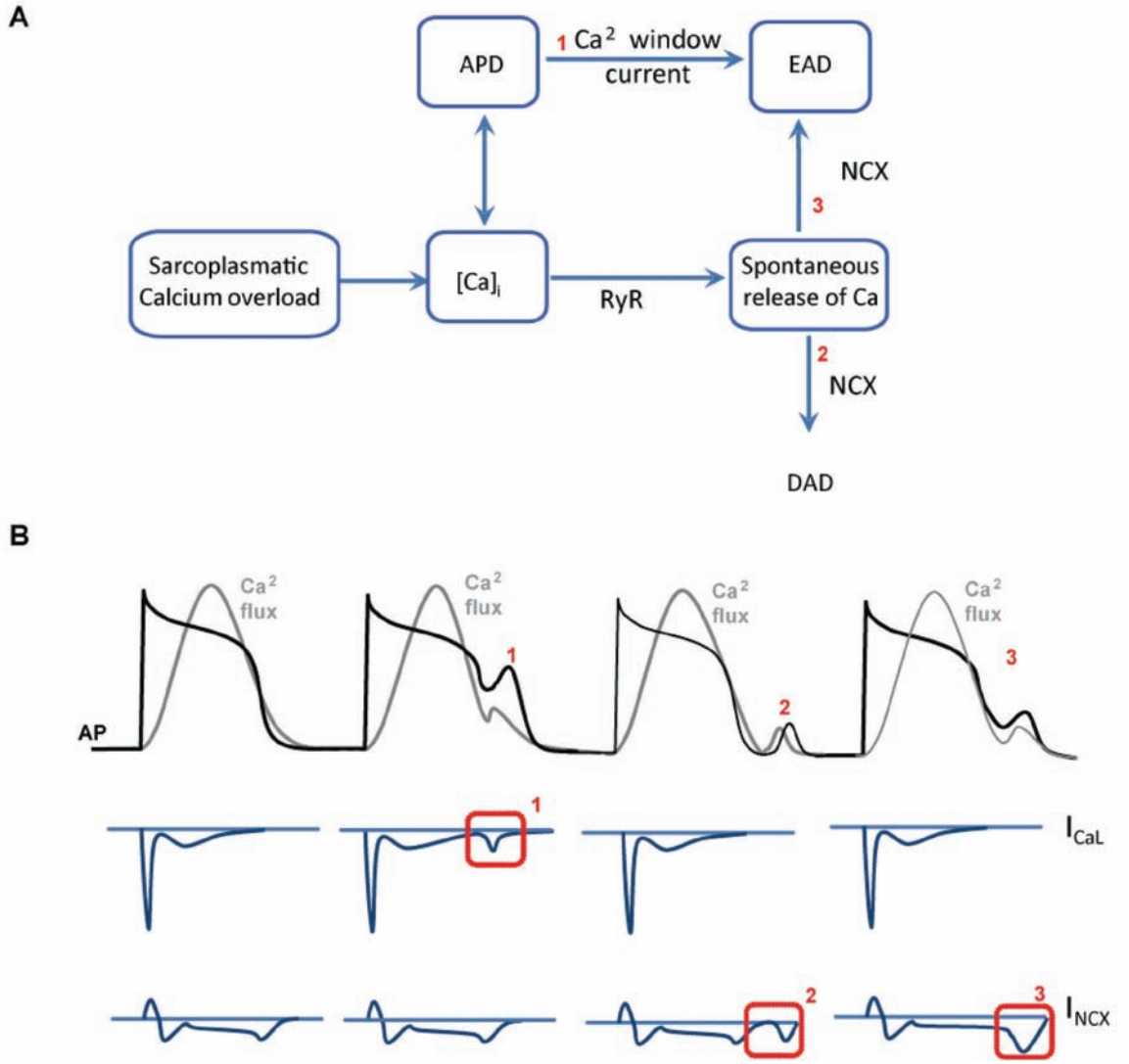

Figure 3. EAD and DAD formation.
A: SR calcium overload leads to increased $[Ca^{2+}]_i$. This can lead to prolonged action potential duration creating a calcium window current potentially leading to EAD (1). increased $[Ca^{2+}]_I$ on the other hand can lead to spontaneous calcium release leading to either an DAD (2) or EAD (3) via NCX. **B:** From left to right; normal action potential and I_{CaL} and I_{NCX}; EAD due to calcium window current via LTCC (1); DAD occurring due to forward NCX activity (2); EAD due to forward NCX activity (3).

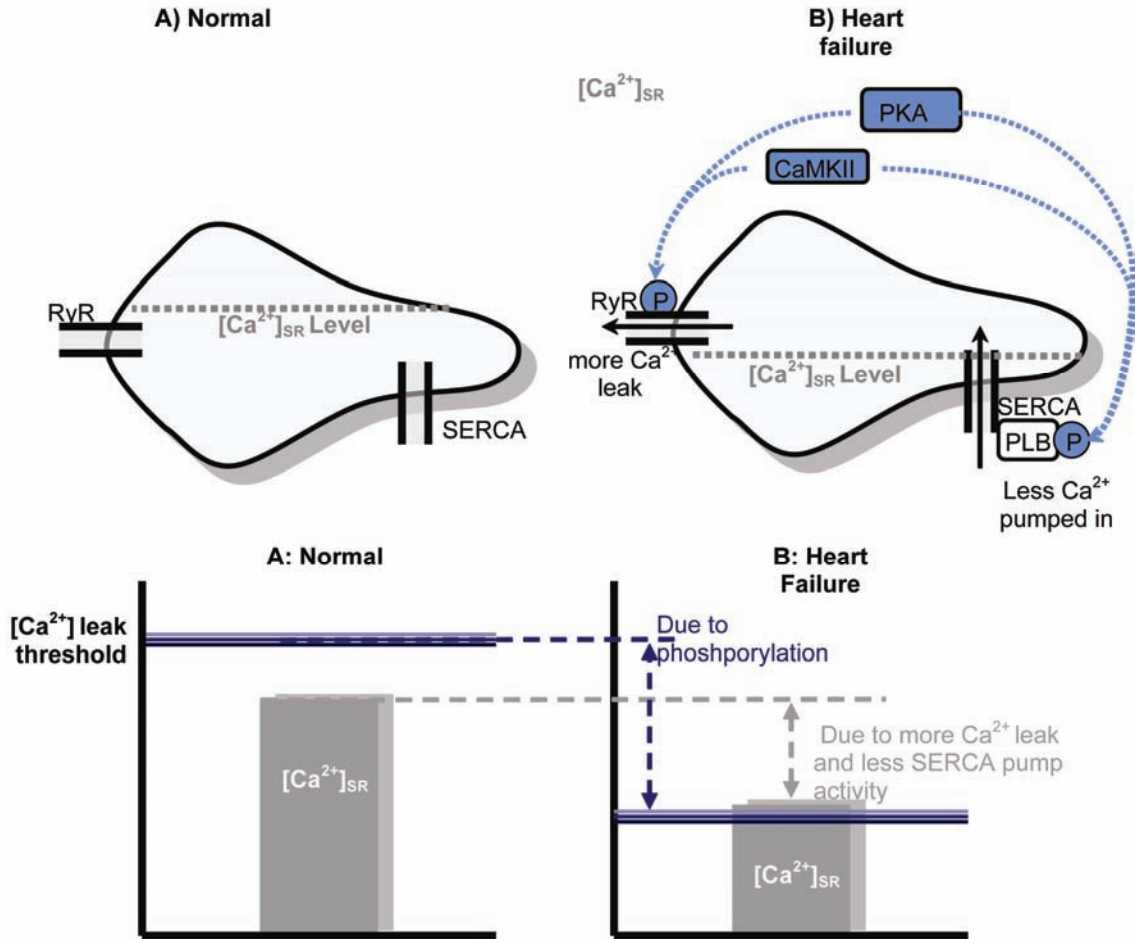

Figure 4. RyR open probability, $[Ca^{2+}]_{SR,}$ and sparks. Calcium sparks occur when $[Ca^{2+}]_{SR}$ reaches RyR opening threshold. RyR opening threshold is influenced by the open probability of RyR. Higher open probability lowers the threshold. $[Ca^{2+}]_{SR}$ is affected by total $[Ca^{2+}]_{in}$ and SERCA. Fig B: In heart failure $[Ca^{2+}]_{SR}$ is lowered but the RyR open threshold is lowered more extensively rendering $[Ca^{2+}]_{SR}$ higher than threshold. The upper part denotes a schematic SR, the lower part a (unquantified) graph.

Conclusion

Every normal heart beat is dependent on well orchestrated conduction on the organic level, homogeneous intercellular coupling, and appropriate excitation on the cellular level. When one or more of these factors is disturbed in its functioning, arrhythmias can occur.
Severe conduction slowing, and especially conduction block, can result in re-entry based arrhythmias, where the electrical signal circulates perpetually. Profound factors influencing conduction are fibrosis, cell size, sodium current, and cell-to-cell coupling via gap junctions.
On the other hand, excitation-based arrhythmias also exist, namely triggered arrhythmias caused by EADs and DADs. Calcium seems to play a pivotal role in these arrhythmias, with LTCC window currents or SR calcium leak, leading to a net depolarizing current once the calcium is removed by the NCX.
From the aforementioned we can conclude that, with the central role of calcium in triggered and (via cardiac remodeling) re-entry based arrhythmias, therapy should logically also focus on calcium. This can be done either by improving existing therapies (class IV antiarrhythmics) or by introducing new treatments, like entities that target the calcium-dependent signaling proteins.

References

1. Ramanathan C, Jia P, Ghanem R, Ryu K, Rudy Y. Activation and repolarization of the normal human heart under complete physiological conditions. *Proc Natl Acad Sci U S A*. 2006;103:6309-6314
2. Jansen JA, van Veen TA, de Bakker JM, van Rijen HV. Cardiac connexins and impulse propagation. *Journal of molecular and cellular cardiology*. 2010;48:76-82
3. Bers DM. Cardiac excitation-contraction coupling. *Nature*. 2002;415:198-205
4. Allessie MA, Bonke FI, Schopman FJ. Circus movement in rabbit atrial muscle as a mechanism of tachycardia. Ii. The role of nonuniform recovery of excitability in the occurrence of unidirectional block, as studied with multiple microelectrodes. *Circulation research*. 1976;39:168-177
5. Jansen JA, Noorman M, Musa H, Stein M, de Jong S, van der Nagel R, Hund TJ, Mohler PJ, Vos MA, van Veen TA, de Bakker JM, Delmar M, van Rijen HV. Reduced heterogeneous expression of cx43 results in decreased nav1.5 expression and reduced sodium current that accounts for arrhythmia vulnerability in conditional cx43 knockout mice. *Heart Rhythm*. 2012;9:600-607
6. Severs NJ. Pathophysiology of gap junctions in heart disease. *Journal of cardiovascular electrophysiology*. 1994;5:462-475
7. Zhao Z, Wen H, Fefelova N, Allen C, Baba A, Matsuda T, Xie LH. Revisiting the ionic mechanisms of early afterdepolarizations in cardiomyocytes: Predominant by ca waves or ca currents? *American journal of physiology. Heart and circulatory physiology*. 2012;302:H1636-1644
8. Volders PG, Vos MA, Szabo B, Sipido KR, de Groot SH, Gorgels AP, Wellens HJ, Lazzara R. Progress in the understanding of cardiac early afterdepolarizations and torsades de pointes: Time to revise current concepts. *Cardiovascular research*. 2000;46:376-392
9. Antoons G, Volders PG, Stankovicova T, Bito V, Stengl M, Vos MA, Sipido KR. Window ca2+ current and its modulation by ca2+ release in hypertrophied cardiac myocytes from dogs with chronic atrioventricular block. *The Journal of physiology*. 2007;579:147-160
10. Antoons G, Willems R, Sipido KR. Alternative strategies in arrhythmia therapy: Evaluation of na/ca exchange as an anti-arrhythmic target. *Pharmacology & therapeutics*. 2012;134:26-42
11. Degrande S, Nixon D, Koval O, Curran JW, Wright P, Wang Q, Kashef F, Chiang D, Li N, Wehrens XH, Anderson ME, Hund TJ, Mohler PJ. Camkii inhibition rescues proarrhythmic phenotypes in the model of human ankyrin-b syndrome. *Heart rhythm : the official journal of the Heart Rhythm Society*. 2012
12. Fink M, Noble PJ, Noble D. Ca(2)(+)-induced delayed afterdepolarizations are triggered by dyadic subspace ca2(2)(+) affirming that increasing serca reduces aftercontractions. *American journal of physiology. Heart and circulatory physiology*. 2011;301:H921-935
13. Pogwizd SM, Bers DM. Cellular basis of triggered arrhythmias in heart failure. *Trends in cardiovascular medicine*. 2004;14:61-66

Chapter 3:

Ventricular electrical remodeling in compensated cardiac hypertrophy

Vincent J.A. Bourgonje, Toon A.B. van Veen, Marc A. Vos

Electrical Diseases of the Heart, 2nd edition - Ihor Gussak, Charles Antzelevitch, Arthur Wilde, Brian Powell, Michael Ackerman, and Win-Kuange Shen

ABSTRACT:

Ventricular hypertrophy is an adaptation of the heart that develops in response to congenital or acquired pathologies to reduce wall stress and to maintain cardiac output. These adaptations can be successful (compensated) or inadequate, leading to deterioration of cardiac function, resulting in heart failure.
A model of compensated hypertrophy is the dog with chronic AV-block (CAVB), in which cardiac remodeling occurs after AV-block, without deterioration towards heart failure.

Electrically, the most notable change is a lengthening of the QT-interval on the ECG. This is due to a lengthening of the action potential, which in turn depends on down regulation of repolarizing potassium currents. This long-QT phenotype leads to increased propensity for Torsade de Pointes (TdP) arrhythmias.
Contractile remodeling is observed in an increased systolic calcium concentration, while diastolic levels are not elevated, leading to improved contraction and increased dP/dT in the left ventricle. The increased calcium transient is linked to an increased loading of the sarcoplasmic reticulum.
Structurally, remodeling is characterized by hypertrophy of both ventricles. However, this is not accompanied by increased fibrosis or decreased intercellular coupling or conduction.
Intracellular signaling in the CAVB dog is reminiscent of exercise-induced hypertrophy. Calcineurin is not activted, while the CaMKII signaling cascade is interrupted at the HDAC4 level. Akt however, likewise in physiological hypertrophy, is activated.
The precise mechanism of arrhythmogenesis in this model is unclear, but could be due to an L-type calcium window current or an increased sodium late current, as inhibition of these currents is anti-arrhythmic. Reentry is probably not involved, as conduction is not slowed. Also, the presence of early afterdepolarizations in isolated cardiomyocytes suggests triggered activity as a leading mechanism instead.

Concluding, the compensated hypertrophy in the CAVB dog is accompanied by electrical remodeling, leading to a severely increased sensitivity for arrhythmias.

INTRODUCTION

Ventricular hypertrophy is an adaptation of the heart that develops in response to either congenital or acquired pathologies in order to reduce wall stress ***(1)*** and to maintain cardiac output. Initially, the term remodeling was reserved for structural changes (hypertrophy, dilatation). More recently, it has been recognized that adaptive mechanisms are present on different levels (structural, contractile, and electrical) ***(2)***. After an insult, these adaptations can be successful (compensated) or inadequate leading to deterioration of cardiac function, resulting in heart failure (Figure 1A). Structurally, the compensated heart has regained the balance between wall thickness and internal cavity dimension ***(3)***, whereas the failing heart becomes quite dilatated in time ***(4)*** (Figure 1B). On the cellular level, there is an increase in the duration of the cellular action potential ***(5)*** (electrical remodeling, Figure 1C) that is accompanied by an increased intracellular calcium transient ***(6)***, or by a severely depressed one ***(7;8)*** (contractile remodeling). In case of the latter, the intracellular diastolic calcium levels ($[Ca^{2+}]_i$) are often elevated too ***(7)*** (Figure 1C). Also the signal transduction pathways involved in the remodeling process may differ ***(9;10)***. It is known that exercise leads to a physiological hypertrophy in which the pAkt pathway is dominant ***(11;12)***, whereas heart failure or pathological hypertrophy is ruled, among others, by the calcineurin ***(13;14)*** and possibly the Ca^{2+}-calmodulin-kinase (CaMKII) pathways ***(15)*** (Figure 2).

Ventricular remodeling is known to increase the propensity for ventricular arrhythmias and may lead to sudden cardiac death. Despite a clear association between left ventricular mechanical dysfunction, hypertrophy and ventricular arrhythmias ***(16)***, the majority of sudden cardiac death occurs at earlier stages of the disease process, even in circumstances in which mechanical dysfunction or ventricular hypertrophy, are absent ***(16-18)***. The mechanisms by which contractile and electrical remodeling predispose to arrhythmias remain unclear, but the changes in $[Ca^{2+}]_i$ handling and repolarization as seen in the compensated form (Figure 1C) may contribute. Therefore, in this chapter, we will concentrate on a canine model of complete atrio-ventricular block (CAVB) in which the beneficial adaptations leading to compensated biventricular hypertrophy are accompanied by a detrimental one, being an increased susceptibility for repolarization related ventricular arrhythmias. We will discuss the specific remodeling changes that are related to this phenotype.

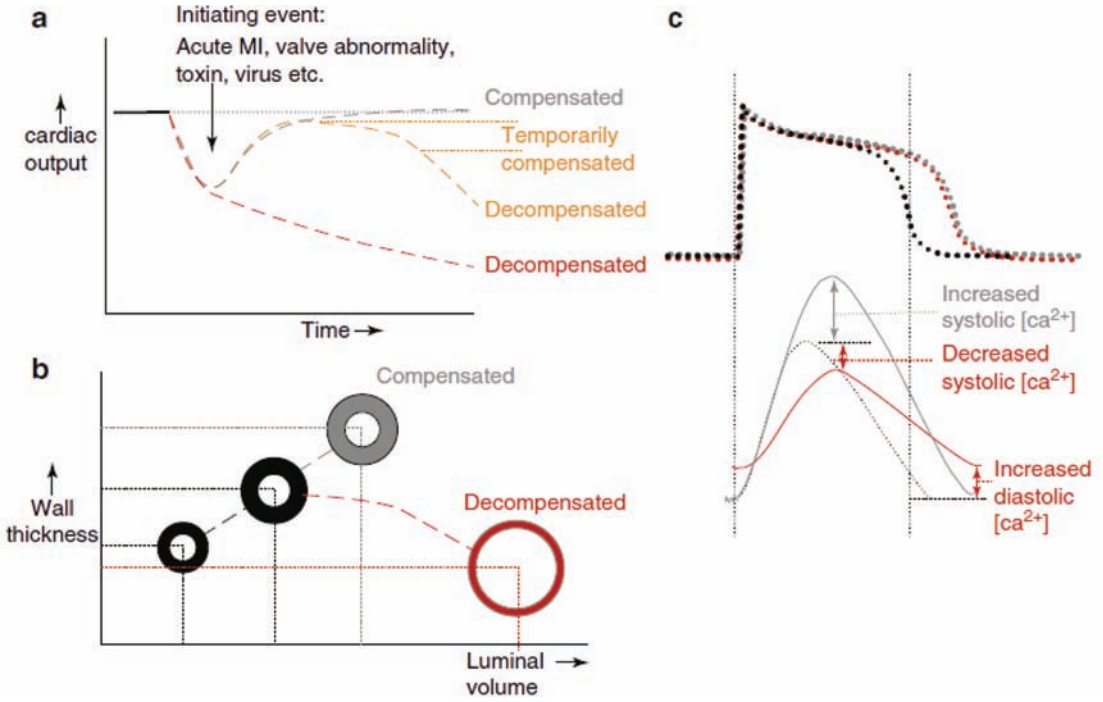

Figure 1: Differences between decompensated and compensated hypertrophy
A: Evolvement of cardiac output over time in compensated (grey line) and decompensated (yellow and red lines) hypertrophy after an initiating event
B: in compensated hypertrophy wall thickness and luminal volume increase (grey circle), while in decompensated hypertrophy dilatation occurs (red circle).
C: Lower part: the differences in intracellular calcium levels at baseline (black) and in compensated (grey) and decompensated (red) hearts. In the upper part the accompanying cardiac action potentials are shown for reference.

VENTRICULAR REMODELING IN THE CAVB DOG

Creation of chronic CAVB by radiofrequency ablation results in numerous adaptations initiated to overcome, acutely and in the long run (weeks), the abrupt decrease in cardiac output (Figure 3A, black line) due to the bradycardia (Figure 3A, red line). The CAVB dog ***(19)*** is a model of compensated biventricular hypertrophy with a long QT phenotype ***(3;6;19;20)***. The primary compensatory parameter is illustrated in the recovery of cardiac

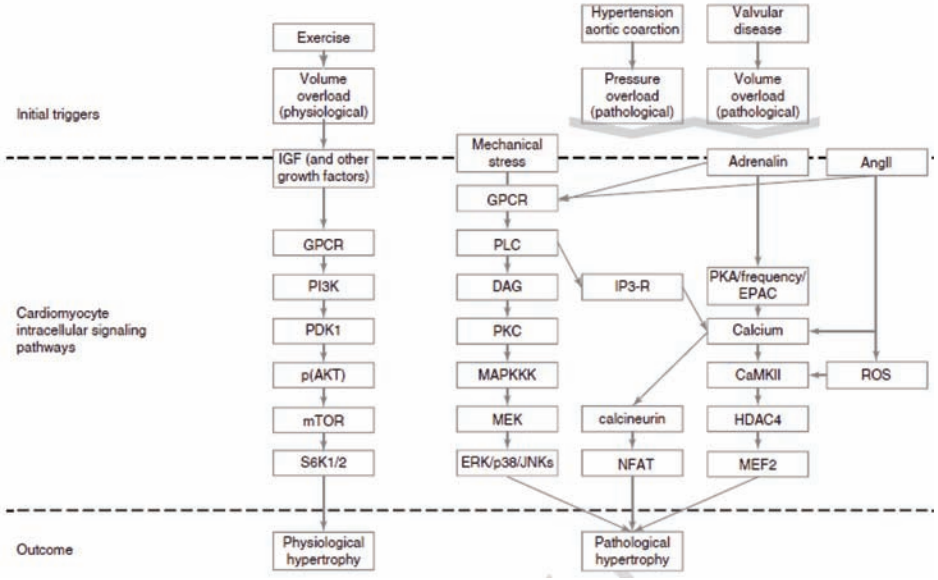

Figure 2: The intracellular signal transduction pathways involved in cardiac hypertrophy leading to either a more physiological or pathological phenotype

output after the initial decline. Initially, this is reached by increasing cardiac contractility as can be seen in left ventricular (LV) pressure development over time (LV dP/dt, Figure 3A, green line). However, as time progresses, a decline is seen, until it reaches a stable state at >10 weeks that accounts for 120% of baseline values. Nonetheless, also on this longer time scale cardiac output is maintained, because biventricular hypertrophy develops (increased heart weight to body weight, Figure 3A, yellow line) creating a new equilibrium between wall thickness and LV volume, which prevents deterioration into dilated cardiomyopathy (for reference: see Figure 1). ***(3;21)***

Aside from the structural and mechanical changes, electrical remodeling is also present. Grossly, this is identified in a lengthening of the cellular action potential duration, that in vivo is reflected in prolongation of the QT-time (Figure 1, blue line) ***(3;7;17;19-21)***. This is pro-arrhythmogenic, as further drug-induced lengthening leads to early afterdepolarizations (EADs), extra beats, and ultimately life threatening Torsades de Pointes arrhythmias (TdP) (Figure 3B). ***(17;19;22-24)***

In the following paragraphs, we will describe in depth the electrical, contractile, and structural remodeling. Moreover, the possible mechanisms responsible for the enhanced arrhythmogeneity (arrhythmogenic outcome) and possible responsible intracellular signaling pathways will be discussed. Special focus will be on the existing intricate connections between ventricular remodeling and their effect on arrhythmogeneity.

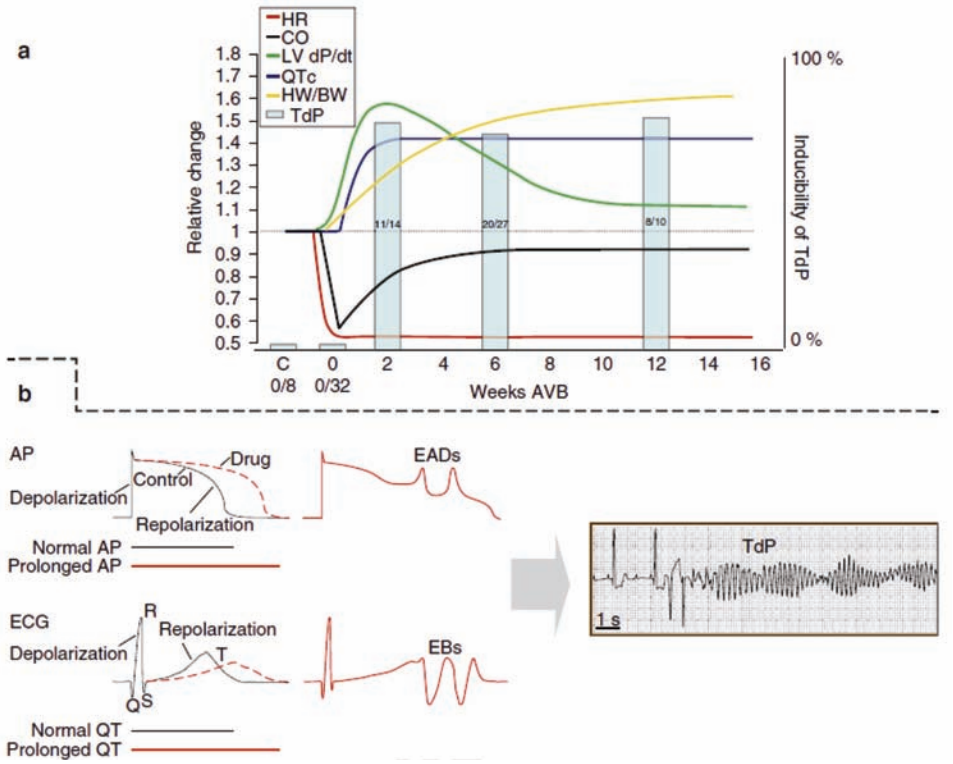

Figure 3: Progress of remodeling in the CAVB dog
A: Schematic figure summarizing the development of cardiac remodeling in the CAVB dog over time. Scale is relative. The red line shows development of heart rate. The black line is an approximation of cardiac output. The green line shows contractile remodeling as left ventricular pressure changes. The yellow line represents structural remodeling, via cardiac hypertrophy as heart weight to body weight. The blue line depicts QT interval lengthening on the ECG, as a parameter for electrical remodeling. Finally, the bars represent fraction of dogs sensitive to drug-induced TdPs (see right Y-axis) at 2, 6, or 12 weeks of chronic AV-block.
B: Summary of the mechanism of drug-induced repolarization-dependent arrhythmias. From lengthening of the QT interval/APD, to early afterdepolarizations (EADs) and extra beats (EBs), to Torsade de Pointes arrhythmias (TdP)

ELECTRICAL REMODELING

As mentioned above, the most striking electrical remodeling is seen in a lengthening of the action potential duration, which can be observed via lengthening of the QT interval on the ECG or, more regionally, via an increase in the duration of the catheter-based monophasic action potentials (MAPD, Table 1A) ***(17;19;22;24)*** or the activation recovery interval (ARI) of a needle measurement ***(25)***. The prolongation of the MAPD is larger in the LV versus the right ventricle (RV), indicating that interventricular dispersion assessed as LV MAPD – RV MAPD is increasing too (Table 1A) ***(17;19;24;25)***. More recently, spatial dispersion was further assessed using 66 needles with 4 electrodes, demonstrating increases in transseptal, interventricular, LV transmural as LV apex to base dispersion ***(25)***. Also temporal dispersion, quantified as short term variability of beat-to-beat repolarization was increased after CAVB ***(24)***. So not only is the repolarization prolonged, but it is also non-uniform in space and time. Lengthening of the QT-duration develops in the first two weeks after AV-block and then stabilizes (Figure 3A, blue line) ***(23)***. Cellular experiments performed at 3-9 weeks of CAVB in isolated cardiomyocytes confirm this increase in repolarization times ***(26;27)***. Since potassium currents are largely responsible for proper repolarization ***(28)***, the most logical assumption would be to expect a decrease in these currents in the CAVB dog. Indeed, both the

slow component of the delayed rectifier current Ik_s, as to a lesser extend the rapid component (Ik_r), are downregulated in the LV and in the RV (,Table 1B) ***(29)***. Other repolarizing currents as the inward rectifying potassium current (Ik_1) and the transient outward current (I_{to}) are not changed functionally in either of the two ventricles. ***(29)***
Also the inward currents are attenuated when compared to their functioning before AV-block. The peak sodium current (I_{Na}) is down in the LV (Table 1B) ***(25)***, whereas I_{Na} late, which occurs in the plateau of the action potential, is also reduced ***(30)***. The current through the L-type calcium current ($I_{Ca\text{-}L}$) does not change after AV-block ***(6)***, but a more frequent occurrence of the window current is observed ***(31)***. Sipido et al ***(6)*** observed an increased current via the sodium calcium exchanger (NCX), in both modes, responsible for a more active sarcolemmal exchange of calcium and natrium ions through this channel. Also this increase may be pro-arrhythmic (see further). In line with this observation, $[Na^+]_i$ seems to be increased, while activity of the Na^+/K^+ exchanger remained similar ***(32)***. The reduction in both peak and late sodium current are not in line with an increase in $[Na^+]_i$ or $[Ca^{2+}]_i$ nor with an increase in cellular APD. Recently we studied the relevance of the sodium-proton exchanger (Na^+/H^+) (unpublished data). The activity of this pump seems elevated thereby possibly explaining the increased $[Na^+]_i$. Taken together, repolarization reserve in the CAVB model has been diminished.
For a complete summary of the observed changes in ion currents in the CAVB dog, see Table 1. On mRNA and protein level, appropriate changes of the (alpha) -subunits of ion channel proteins have been observed ***(26;33)***. A reference for the role of ion currents in a normal action potential can be found in Figure 4A.

Reference	QT	MAPD	Spatial dispersion	Temporal dispersion
Vos et al. [19]	↑	↑	↑	
Schoenmakers et al. [17]	↑	↑	↑	
Boulaksil et al. [25]	↑	↑(ARI)	↑(ARI)	
Thomsen et al. [24]	↑	↑	↑	↑
Dunnink et al. [22]	↑	↑	↑	↑

Reference	Etiology	INa	ICaL	Ito	Ik_r	Ik_s	Ik1	NCX	Na/K pump
Sipido et al. [6]	CAVB		=	=			=	↑ ↑	
Volders et al. [29]	CAVB			=.	= =	↓ ↓	↓ =.	=	
Boulaksil et al. [25]	CAVB	↓(peak) =							
Antoons et al. [30]	CAVB	↓ Late							
Antoons et al. [31]	CAVB		Window current						
Stengl et al. [26]	CAVB					↓			
Verdonck et al. [32]									=

Table 1: Characterization of electrical remodeling in the CAVB dog
Upper: Summary of studies which report APD lengthening and variability, via monophasic action potential duration (MAPD) or QT interval lengthening on the ECG in AV-block dogs
Lower: Summary of the ion current changes observed in the CAVB dog

CONTRACTILE REMODELING

The ion current responsible for coupling the electrical impulse to contraction is I_{CaL} via the L-type calcium channel (LTCC) ***(34)*** (Figure 4B), which is voltage-sensitive and activates upon depolarization. The ryanodine receptor (RyR), located at the sarcoplasmic reticulum (SR), opens in accordance with the influx of calcium through LTCC, and even more calcium will be released in the cytoplasm; a process called 'calcium-induced calcium release' ***(34)***. The SR functions as an intracellular calcium store. Cytosolic calcium binds to the myofilaments and

myocyte shortening/contraction occurs. Thereafter $[Ca^{2+}]_i$ returns to baseline values with the assistance of the NCX, transporting calcium out of the cell, while the (larger) remainder of cytoplasmic Ca^{2+} is pumped back into the SR via the sarcoplasmic reticulum calcium ATPase (SERCA). This is summarized in figure 4B and in the left part of figure 4C. Also, for a more detailed review of exitation-contraction coupling, see Bers. ***(34)***

Contractile strength can be varied under regulation of adrenergic stimuli, like epinephrine. The ß-adrenergic pathway modifies contraction by phosphorylation of a number of key proteins: 1) LTCC, 2) RyR release, and 3) SR reuptake of calcium by phosphorylation of phospholamban, the ancillary inhibitor protein of SERCA. ***(34)*** Also, CamKII has a positive, facilitating function in increasing contraction by phosphorylation of the same set of proteins, although at a different amino-acid residue. ***(35)***

In the CAVB dog, the $[Ca^{2+}]_i$ transient is longer and in amplitude increased (Figure 1C), while diastolic calcium levels are unaltered, thereby enhancing the systolic calcium fluxes as compared to non-hypertrophied, non-remodeled cardiomyocytes ***(6)***. These increased $[Ca^{2+}]_i$ levels result in more contractile power at the slow heart rhythm (bradycardia) in this animal model. A negative force frequency relationship occurs (in both ventricles) ***(6;20)***, which is in contrast to normal physiological behavior ***(7;36)***. Also, potentiation of contraction, as achieved by extra-stimuli, is increased in the CAVB dog ***(20)***. As mentioned before, both the Na^+/ Ca^{2+} and possibly Na^+/H^+ exchange, and SERCA have increased functional activity in order to handle this larger sarcolemmal and SR calcium movements. There is a close relation between electrical and contractile remodeling (see arrhythmogenic outcome).

The stronger contractility can be measured in vivo using LV or RV dP/dt+, which is clearly increased at 2 weeks of CAVB (Figure 3A, green line) ***(20)***. Neurohumoral activation as seen in temporarily increased levels of (nor) epinephrine, angiotensin II and aldosteron in the blood plasma of these dogs is in agreement. Both contractility and neurhormonal leves are transiently increased, since after 4-6 weeks of CAVB, all measured neurohumoral plasma concentrations are back to baseline ***(19;26)***.

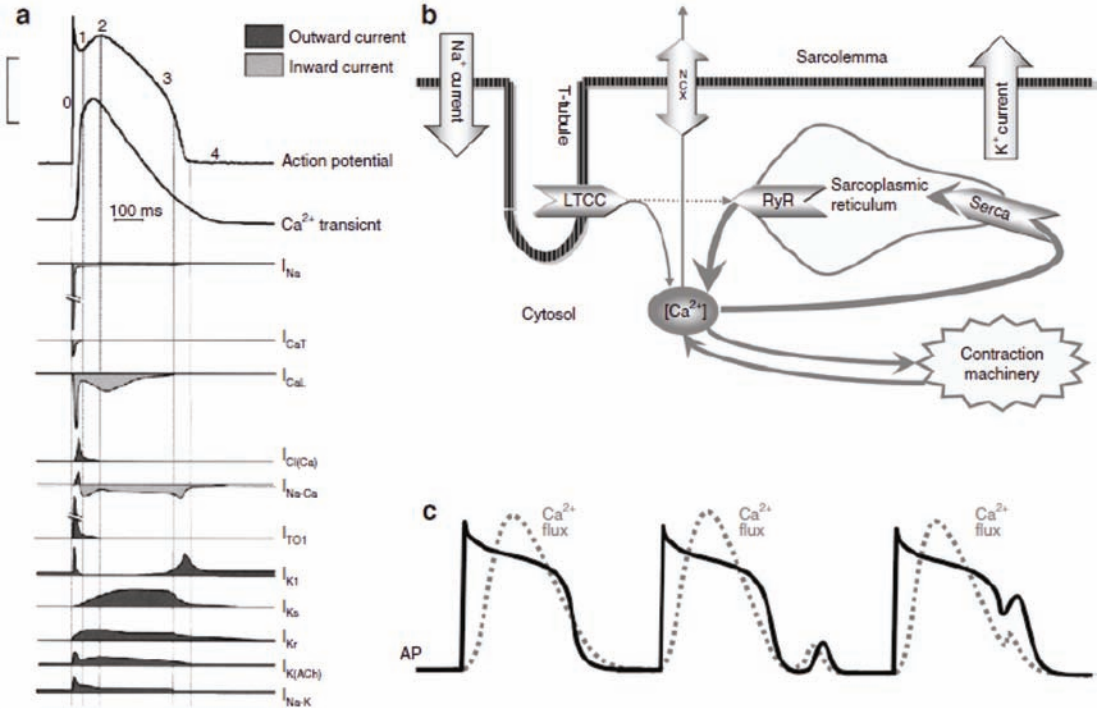

Figure 4: Ion currents and calcium handling in the cardiomyocyte
A: Schematic illustration of the depolarizing and repolarizing currents that shape the action potential in the normal mammalian ventricle. Time course of each of the currents is shown together with the course of the Ca2+ transient.
B: Depiction of intracellular calcium movement during an action potential. Upon depolarization the L-type calcium channel opens (LTCC) and a relatively small amount of calcium enters the cardiomyocyte. The ryanodine receptor (RyR) reacts to this calcium and opens as well, leading to release of calcium out of the sarcoplasmatic reticulum. The myofilaments start contracting in response to the increased calcium concentration. At the end of the action potential the intracellular calcium concentration is reduced to baseline by the sarcoplasmic reticulum calcium-ATPase (SERCA) pump, which pumps calcium back in to the sarcoplasmic reticulum, and via the sodium/calcium exchanger (NCX), which removes a smaller amount of calcium in exchange for sodium ions (3 sodium ions for 1 calcium ion).
C: Behaviour of the intracellular calcium flux during a normal action potential, and a delayed and early afterdepolarization. The dotted line depicts the intracellular calcium concentration. Note that a delayed afterdepolarization occurs after the end of the action potential, while a early afterdepolarization occurs during the repolarization phase of an action potential.

STRUCTURAL REMODELING

The most obvious structural change is of course the biventricular hypertrophy. This can be seen on the whole heart level as an increase in the heart to body weight (Figure 5A), as well as on LV and RV weight determinations ***(19)***, as on the cellular level, where the cardiomyocytes are both lengthened and broadened ***(3;25;27)*** (Figure 5A). In this animal model, hypertrophy is more pronounced in the RV than in the LV as is reflected in the averaged increase in the length of the individual cardiomyocytes of Figure 5A.
Profound hypertrophy, especially of the pathological kind, can be accompanied by extensive fibrosis, decoupling of the cardiomyocytes and slowing of the conduction velocity ***(37;38)***. In the CAVB dog, neither is present: interstitial fibrosis, quantified using Sirius red staining, does not increase (Figure 5B), nor is there a decrease in connexin43, a gap junction protein that constitutes channels responsible for ventricular electrical coupling (Figure 5C) ***(37)***. Finally, the capillary-fiber ratio of the myocytes remains similar ***(19)***. As a consequence, CAVB does not affect electrical conduction over the myocardium (Figure 5D) ***(25)***.

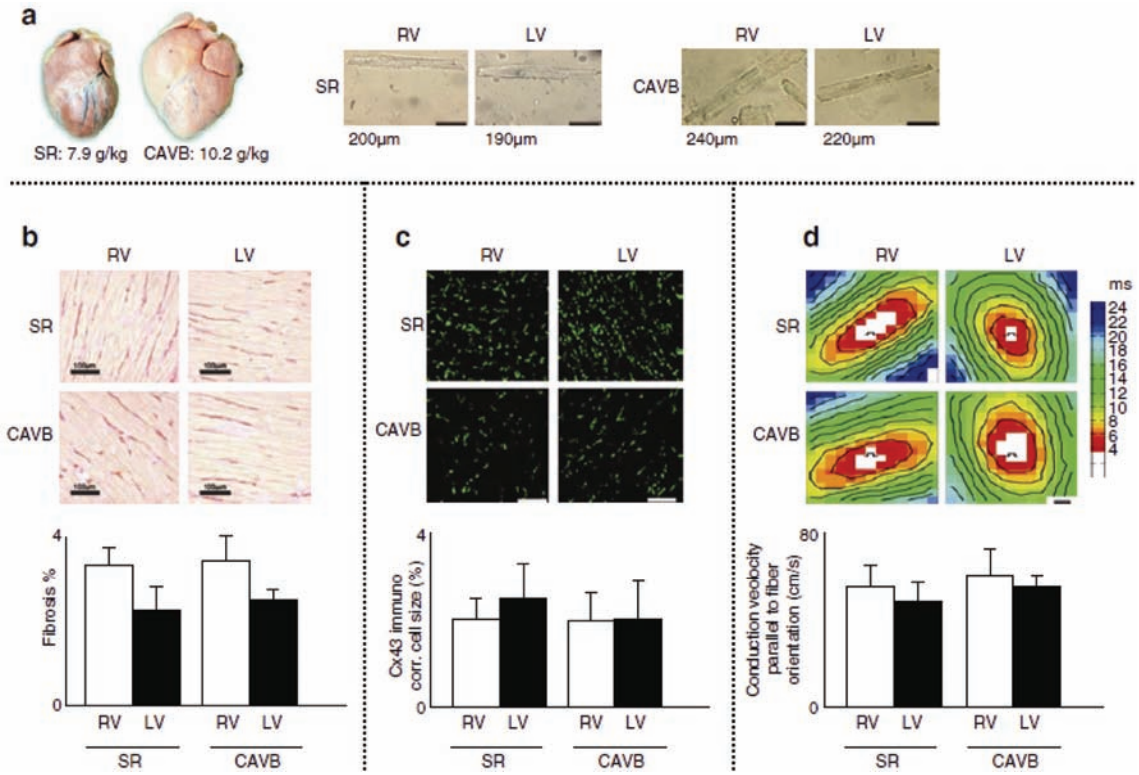

Figure 5: Structural remodeling in the CAVB dog and its consequences for conduction
A: Cardiac hypertrophy in the CAVB dog heart and at the cellular level
B: Comparison of ventricular fibros in sinus (SR) rhythm and chronic AV-block (CAVB) dogs as assessed by Sirius red staining
C: Comparision of Connexin43 (Cx43) expression level and distribution in SR and CAVB dogs as assessed by immunohistochemistry.
D: measurement of ventricular impulse propagation, both in SR and CAVB dogs via epicardial mapping. Red depicts early activation, blue late. The pacing site (indicated with the pacing symbol) was from the center of the epicardial placed electrode grid.

ARRHYTHMOGENIC OUTCOME

The enhanced susceptibility of this animal model for drug induced Torsade de Pointes arrhythmias (Figure 3B) indicates that the beneficial adaptations resulting in compensated biventricular hypertrophy have deleterious effects on electrophysiology and ventricular repolarization. Especially, repolarization reserve is severely diminished in these animals. Generally, there are roughly two ways in which arrhythmias can develop: reentry or triggered-activity ***(39)***. Reentry based arrhythmias are dependent on conduction slowing and unidirectional block of conduction, circumstances which promote self-sustaining electrical wavefronts. This mechanism of arrhythmogeneity can probably be excluded in case of the CAVB dog, as the necessities for these kind of arrhythmias are not present, which is most clearly shown by its retained conduction velocity (see figure 5D), and inability to demonstrate contribution of reentry in the initiation and perpetuation of TdPs ***(25)***.
Triggered activity either resulting from delayed (DADs, Figure 4B, middle panel) or early afterdepolarizations (EADs, Figure 4B, right panel) are likely involved in the initiation of ectopic beats and ventricular arrhythmias ***(40)***. In this model, their occurrence, alone or in combination, has been demonstrated in numerous conditions, both in vivo ***(19;20)***, as in isolated cells ***(27;41)***. There is a close relation with the excitation-contraction activity, as can

be seen in figure 4B: spontaneous release of Ca^{2+} from the (overloaded) SR through RyR can (re)trigger depolarization of the action potential, either after (delayed) or within (early) the AP. The sequence is now reversed to mechanical-electrical coupling, as calcium release induces a change in membrane potential, instead of the opposite. In case of DADs, the NCX is most likely responsible for the transient inward current (I_{ti}) ***(42)***. For EADs, window currents carried by the LTCC have been mentioned to underlie these events ***(31)***, probably assisted in a conditional phase induced by the NCX. Especially in conditions when intracellular Ca^{2+}-handling is combined with a decreased repolarization reserve, these interactions may cause this arrhythmogenic mechanism. The drug-induced TdPs as observed, are both initiated and perpetuated by EADs, and triggered ectopic beats, which accumulate to self terminating polymorphic ventricular tachyarrhythmias (Figure 3B, right panel), as we have recently shown in this model ***(25)***.
Controlling the $[Ca^{2+}]_i$ is anti-arrhythmic and block of the LTCC, by verapamil or flunarizine, is clearly effective in preventing and suppressing these arrhythmias in vivo and on the cellular level ***(43)***.

This arrhythmogenic outcome is stable over time (Figure 3, the bars) in the CAVB dog: 60-80% of the dogs show reproducible TdPs, at 2, 6, or 12 weeks after AV-block, whereas no TdP can be induced in dogs without cardiac remodeling, like dogs still in sinus rhythm or directly after AV-block. When evaluating the different remodeling processes (structural, contractile, and electrical), it is clear that electrical remodeling is the only one that remains stable in the weeks after CAVB (Figure 3A). Structural remodeling develops more slowly, whereas contractile adaptations after being profoundly increased transiently, are returning to less increased values in time. ***(23)***

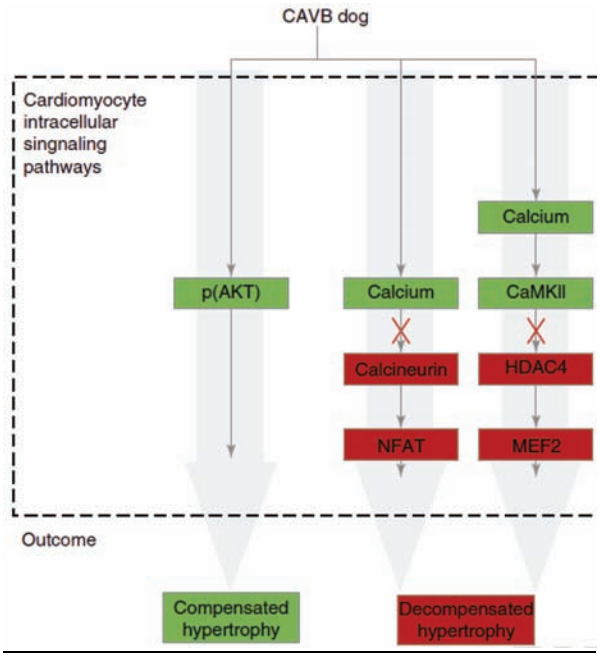

Figure 6: Hypertrophic signaling in the CAVB dog. Pathways involved in decompensated hypertrophy were not activated while the phospho-Akt pathway involved in the compensated phenotype was.

INTRACELLULAR SIGNALING

As has been summarized in figure 2, hypertrophic remodeling is accompanied by activation of a number of signaling pathways in the cardiomyocyte. Of these, some, like calcineurin ***(13;14)*** and CaMKII ***(15)*** (in the heart, CaMKII almost always refers to the CaMKIIδ(delta) variant, as this is the most expressed isoform in the heart) ***(44)***, are related to heart failure, while others, like Akt ***(11;12)***, are more closely linked to physiological hypertrophy. In the CAVB model, mechanotransduction seems to play an prominent role ***(3)***. Besides bradycardia, volume overload and altered ventricular activation are important in generating this different phenotype ***(45)***.

As the CAVB dog has a compensated hypertrophy, one would expect to indentify signaling reminiscent of physiological hypertrophy. This is indeed the case, as Akt was shown to be activated ***(3)***. In contrast, calcineurin, another signaling molecule involved in pathological remodeling, appeared not to be involved in cardiac remodeling of the CAVB dog, which was assessed via calcineurin inhibition through chronic cyclosporin A treatment ***(46)***. Cyclosporin A did not affect electrical, contractile, or structural remodeling, thereby suggesting no role of calcineurin.

CaMKII activation in the dog was more paradoxical. We have recently established that CaMKII total levels were not changed, but autophosphorylation levels were increased. This is indicative for increased CaMKII activity in the CAVB dog. However, the CaMKII-dependent pathway that leads to MEF2-dependent changes in gene expression was not activated, as HDAC4, the link between MEF2 and CaMKII ***(47)***, was not phosphorylated. On the other hand, CaMKII also phosphorylates numerous targets involved in intracellular calcium handling, like RyR, LTCC, and phospholamban (the inhibitor of SERCA) ***(48)***. This implies a regulatory role of CamKII in $[Ca^{2+}]_i$ handling, but no involvement in alterations of the gene expression profile associated with maladaptive remodeling. A summary of the involved signaling pathways in the CAVB dog, as we have observed, can be seen in figure 6.

CONCLUSION:

The CAVB dog has a heart with profound adaptations on the structural, electrical, and contractile levels. This remodeling is compensatory, as the cardiac output is retained in the long-term due to stable biventricular hypertrophy and increased contractility. However, the action potential is heterogeneously lengthened, both in space and time, which is pro-arrhythmic and maladaptive, as the remodeled heart appears much more sensitive to EADs, extra beats, and TdP arrhythmias.

Reference List

(1) Grossman W, Jones D, McLaurin LP. Wall stress and patterns of hypertrophy in the human left ventricle. *J Clin Invest* 1975; 56(1):56-64.

(2) Cohn JN, Ferrari R, Sharpe N. Cardiac remodeling--concepts and clinical implications: a consensus paper from an international forum on cardiac remodeling. Behalf of an International Forum on Cardiac Remodeling. *J Am Coll Cardiol* 2000; 35(3):569-582.

(3) Donker DW, Volders PG, Arts T, Bekkers BC, Hofstra L, Spatjens RL et al. End-diastolic myofiber stress and ejection strain increase with ventricular volume overload--Serial in-vivo analyses in dogs with complete atrioventricular block. *Basic Res Cardiol* 2005; 100(4):372-382.

(4) Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol* 2006; 7(8):589-600.

(5) Bailly P, Benitah JP, Mouchoniere M, Vassort G, Lorente P. Regional alteration of the transient outward current in human left ventricular septum during compensated hypertrophy. *Circulation* 1997; 96(4):1266-1274.

(6) Sipido KR, Volders PG, de Groot SH, Verdonck F, Van de WF, Wellens HJ et al. Enhanced Ca(2+) release and Na/Ca exchange activity in hypertrophied canine ventricular myocytes: potential link between contractile adaptation and arrhythmogenesis. *Circulation* 2000; 102(17):2137-2144.

(7) Davies CH, Harding SE, Poole-Wilson PA. Cellular mechanisms of contractile dysfunction in human heart failure. *Eur Heart J* 1996; 17(2):189-198.

(8) Mulieri LA, Hasenfuss G, Leavitt B, Allen PD, Alpert NR. Altered myocardial force-frequency relation in human heart failure. *Circulation* 1992; 85(5):1743-1750.

(9) Bernardo BC, Weeks KL, Pretorius L, McMullen JR. Molecular distinction between physiological and pathological cardiac hypertrophy: experimental findings and therapeutic strategies. *Pharmacol Ther* 2010; 128(1):191-227.

(10) Balakumar P, Jagadeesh G. Multifarious molecular signaling cascades of cardiac hypertrophy: can the muddy waters be cleared? *Pharmacol Res* 2010; 62(5):365-383.

(11) DeBosch B, Treskov I, Lupu TS, Weinheimer C, Kovacs A, Courtois M et al. Akt1 is required for physiological cardiac growth. *Circulation* 2006; 113(17):2097-2104.

(12) Sussman MA, Volkers M, Fischer K, Bailey B, Cottage CT, Din S et al. Myocardial AKT: the omnipresent nexus. *Physiol Rev* 2011; 91(3):1023-1070.

(13) Bourajjaj M, Armand AS, da Costa Martins PA, Weijts B, van der NR, Heeneman S et al. NFATc2 is a necessary mediator of calcineurin-dependent cardiac hypertrophy and heart failure. *J Biol Chem* 2008; 283(32):22295-22303.

(14) Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J et al. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* 1998; 93(2):215-228.

(15) Anderson ME, Brown JH, Bers DM. CaMKII in myocardial hypertrophy and heart failure. *J Mol Cell Cardiol* 2011; 51(4):468-473.

(16) Tomaselli GF, Beuckelmann DJ, Calkins HG, Berger RD, Kessler PD, Lawrence JH et al. Sudden cardiac death in heart failure. The role of abnormal repolarization. *Circulation* 1994; 90(5):2534-2539.

(17) Schoenmakers M, Ramakers C, van Opstal JM, Leunissen JD, Londono C, Vos MA. Asynchronous development of electrical remodeling and cardiac hypertrophy in the complete AV block dog. *Cardiovasc Res* 2003; 59(2):351-359.

(18) Zipes DP, Wellens HJ. Sudden cardiac death. *Circulation* 1998; 98(21):2334-2351.

(19) Vos MA, de Groot SH, Verduyn SC, van der ZJ, Leunissen HD, Cleutjens JP et al. Enhanced susceptibility for acquired torsade de pointes arrhythmias in the dog with chronic, complete AV block is related to cardiac hypertrophy and electrical remodeling. *Circulation* 1998; 98(11):1125-1135.

(20) de Groot SH, Schoenmakers M, Molenschot MM, Leunissen JD, Wellens HJ, Vos MA. Contractile adaptations preserving cardiac output predispose the hypertrophied canine heart to delayed afterdepolarization-dependent ventricular arrhythmias. *Circulation* 2000; 102(17):2145-2151.

(21) Verduyn SC, Ramakers C, Snoep G, Leunissen JD, Wellens HJ, Vos MA. Time course of structural adaptations in chronic AV block dogs: evidence for differential ventricular remodeling. *Am J Physiol Heart Circ Physiol* 2001; 280(6):H2882-H2890.

(22) Dunnink A, van Opstal JM, Oosterhoff P, Winckels SK, Beekman JD, van der NR et al. Ventricular remodelling is a prerequisite for the induction of dofetilide-induced torsade de pointes arrhythmias in the anaesthetized, complete atrio-ventricular-block dog. *Europace* 2011.

(23) Oros A, Beekman JD, Vos MA. The canine model with chronic, complete atrio-ventricular block. *Pharmacol Ther* 2008; 119(2):168-178.

(24) Thomsen MB, Oros A, Schoenmakers M, van Opstal JM, Maas JN, Beekman JD et al. Proarrhythmic electrical remodelling is associated with increased beat-to-beat variability of repolarisation. *Cardiovasc Res* 2007; 73(3):521-530.

(25) Boulaksil M, Jungschleger JG, Antoons G, Houtman MJ, de Boer TP, Wilders R et al. Drug-Induced Torsade de Pointes Arrhythmias in the Chronic AV Block Dog Are Perpetuated by Focal Activity. *Circ Arrhythm Electrophysiol* 2011; 4(4):566-576.

(26) Stengl M, Ramakers C, Donker DW, Nabar A, Rybin AV, Spatjens RL et al. Temporal patterns of electrical remodeling in canine ventricular hypertrophy: focus on IKs downregulation and blunted beta-adrenergic activation. *Cardiovasc Res* 2006; 72(1):90-100.

(27) Volders PG, Sipido KR, Vos MA, Kulcsar A, Verduyn SC, Wellens HJ. Cellular basis of biventricular hypertrophy and arrhythmogenesis in dogs with chronic complete atrioventricular block and acquired torsade de pointes. *Circulation* 1998; 98(11):1136-1147.

(28) Nabauer M, Kaab S. Potassium channel down-regulation in heart failure. *Cardiovasc Res* 1998; 37(2):324-334.

(29) Volders PG, Sipido KR, Vos MA, Spatjens RL, Leunissen JD, Carmeliet E et al. Downregulation of delayed rectifier K(+) currents in dogs with chronic complete atrioventricular block and acquired torsades de pointes. *Circulation* 1999; 100(24):2455-2461.

(30) Antoons G, Oros A, Beekman JD, Engelen MA, Houtman MJ, Belardinelli L et al. Late na(+) current inhibition by ranolazine reduces torsades de pointes in the chronic atrioventricular block dog model. *J Am Coll Cardiol* 2010; 55(8):801-809.

(31) Antoons G, Volders PG, Stankovicova T, Bito V, Stengl M, Vos MA et al. Window Ca2+ current and its modulation by Ca2+ release in hypertrophied cardiac myocytes from dogs with chronic atrioventricular block. *J Physiol* 2007; 579(Pt 1):147-160.

(32) Verdonck F, Volders PG, Vos MA, Sipido KR. Increased Na+ concentration and altered Na/K pump activity in hypertrophied canine ventricular cells. *Cardiovasc Res* 2003; 57(4):1035-1043.

(33) Ramakers C, Vos MA, Doevendans PA, Schoenmakers M, Wu YS, Scicchitano S et al. Coordinated down-regulation of KCNQ1 and KCNE1 expression contributes to reduction of I(Ks) in canine hypertrophied hearts. *Cardiovasc Res* 2003; 57(2):486-496.

(34) Bers DM. Cardiac excitation-contraction coupling. *Nature* 2002; 415(6868):198-205.

(35) Maier LS, Bers DM. Role of Ca2+/calmodulin-dependent protein kinase (CaMK) in excitation-contraction coupling in the heart. *Cardiovasc Res* 2007; 73(4):631-640.

(36) Hasenfuss G, Holubarsch C, Hermann HP, Astheimer K, Pieske B, Just H. Influence of the force-frequency relationship on haemodynamics and left ventricular function in patients with non-failing hearts and in patients with dilated cardiomyopathy. *Eur Heart J* 1994; 15(2):164-170.

(37) Boulaksil M, Winckels SK, Engelen MA, Stein M, van Veen TA, Jansen JA et al. Heterogeneous Connexin43 distribution in heart failure is associated with dispersed conduction and enhanced susceptibility to ventricular arrhythmias. *Eur J Heart Fail* 2010; 12(9):913-921.

(38) Wolk R. Arrhythmogenic mechanisms in left ventricular hypertrophy. *Europace* 2000; 2(3):216-223.

(39) Rosen MR. Mechanisms for arrhythmias. *Am J Cardiol* 1988; 61(2):2A-8A.

(40) Weiss JN, Garfinkel A, Karagueuzian HS, Chen PS, Qu Z. Early afterdepolarizations and cardiac arrhythmias. *Heart Rhythm* 2010; 7(12):1891-1899.

(41) Nalos L, Varkevisser R, Jonsson M, Houtman M, Beekman J, van der Nagel R et al. Comparison of I(Kr) blocking drugs Moxifloxacin and Dofetilide/E-4031 in 5 screening models of pro-arrhythmia reveals insufficient specificity of isolated cardiomyocytes. *Br J Pharmacol* 2011. Epub ahead of print

(42) Volders PG, Kulcsar A, Vos MA, Sipido KR, Wellens HJ, Lazzara R et al. Similarities between early and delayed afterdepolarizations induced by isoproterenol in canine ventricular myocytes. *Cardiovasc Res* 1997; 34(2):348-359.

(43) Oros A, Houtman MJ, Neco P, Gomez AM, Rajamani S, Oosterhoff P et al. Robust anti-arrhythmic efficacy of verapamil and flunarizine against dofetilide-induced TdP arrhythmias is based upon a shared and a different mode of action. *Br J Pharmacol* 2010; 161(1):162-175.

(44) Tombes RM, Faison MO, Turbeville JM. Organization and evolution of multifunctional Ca(2+)/CaM-dependent protein kinase genes. *Gene* 2003; 322:17-31.

(45) Winckels SK, Thomsen MB, Oosterhoff P, Oros A, Beekman JD, Attevelt NJ et al. High-septal pacing reduces ventricular electrical remodeling and proarrhythmia in chronic atrioventricular block dogs. *J Am Coll Cardiol* 2007; 50(9):906-913.

(46) Bourgonje VJA, Schoenmakers M, Beekman HDM, Van Der Nagel R, De Windt LJ, Van Veen AAB et al. Discrepancy between acute and long-term effects of the calmodulin/CaMKII/calcineurin pathway on arrhythmogenesis in the CAVB dog. *European Heart Journal* 2010; 31:77-78.

(47) Zhang T, Kohlhaas M, Backs J, Mishra S, Phillips W, Dybkova N et al. CaMKIIdelta isoforms differentially affect calcium handling but similarly regulate HDAC/MEF2 transcriptional responses. *J Biol Chem* 2007; 282(48):35078-35087.

(48) Anderson ME. Multiple downstream proarrhythmic targets for calmodulin kinase II: moving beyond an ion channel-centric focus. *Cardiovasc Res* 2007; 73(4):657-666.

Chapter 4:

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

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Abstract:

Background:
The calcium-dependent signaling molecules calcineurin and CaMKII have both been linked to decompensated hypertrophy and arrhythmias. CaMKII is also believed to be involved in acute modulation of ion channels.

Objective:
To determine the role of calcineurin and CaMKII in a dog model of compensated hypertrophy and a long-QT phenotype.

Methods and Results:
AV-block was created in dogs to induce ventricular remodeling, including enhanced susceptibility to dofetilide-induced Torsades de Pointes arrhythmias (TdP). Dogs were treated with cyclosporin A for three weeks, which reduced calcineurin activity, as determined by mRNA expression levels of *regulator of calcineurin 1 exon 4*, but was unable to prevent structural, contractile, or electrical remodeling and arrhythmias. Biopsies were taken, before, and 2 or 9 weeks after AV-block. Western blots were performed against phosphorylated and total CaMKII, phospholamban, Akt, and histone deacytelase 4 (HDAC4). CAVB showed an increase in Akt, CaMKII and phospholamban phosphorylation levels, but HDAC4 phosphorylation remained unaltered. Dofetilide induced TdP in vivo and early afterdepolarizations in cardiomyocytes, and also increased $[Ca^{2+}]_i$ and CaMKII autophosphorylation. Both W-7 and KN-93 treatment counteracted this.

Conclusions:
The calcineurin pathway seems not involved in the long-term cardiac remodeling of the CAVB dog. Although CaMKII is chronically activated, this is not translated to HDAC4-phosphorylation. However, acute CaMKII overactivation was able to initiate arrhythmias based on triggered activity.

Abbreviations:
CaMKII = Calcium/calmodulin dependent protein kinase II
CAVB = chronic AV-block
TdP = Torsades de Pointes
CSA = Cyclosporin A
EADs = early afterdepolarizations
Rcan1-4 = Regulator of calcineurin 1 exon 4
HDAC4= Histone deacetylase 4
AP(D) = action potential (duration)
STV = Short term variability
MEF2 = Myocyte enhancer factor 2

Introduction:

Cardiac remodeling can result in a compensated or decompensated (heart failure) phenotype. In both conditions, remodeling predisposes for arrhythmias.
In the failing human heart, expression of the calcium-sensitive signaling molecules CaMKII[1, 2] and calcineurin-A (CnA) is increased[3]. Also, in mice overexpressing CnA[4] 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 knock-out mice subjected to pressure overload preserved cardiac function[8].
Acutely activated CaMKII can also directly phosphorylate ion channels, modulate excitation and contraction, and induce arrhythmias[9]. Therefore, CaMKII-induced pro-arrhythmic modulation can be subdivided between acute and chronic processes[10], which both can be investigated through pharmacological 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 CAVB dog model, including elevated calcium levels. Other remodeling processes as prolonged QT-interval and dispersed APD, and bi-ventricular hypertrophy) have been described too, resulting in a high susceptibility for TdP arrhythmias after acute administration of the I_{Kr} blocker dofetilide. In line with this, isolated CAVB cardiomoycoytes were susceptible to EADs after dofetilide infusion.
In the CAVB dog, remodeling results in a preserved ventricular function. We hypothesized that in the CAVB dog model, CaMKII and calcineurin, as mirrored to their role in heart failure, would differentially contribute to cardiac remodelling and arrhythmogenesis, which was assessed by pharmacologic modulation of CaMKII and calcineurin, in vivo and in vitro.

Methods:

22 dogs (25±4kg, 63% ♀) were used in this study using 6 different protocols (A-F). Three experimental procedures (protocol A/B/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 under general anesthesia and AV-block was induced via radiofrequency ablation. Specific details concerning anesthesia, AV-node ablation, TdP characteristics and instrumentation can be found in the supplemental methods.In this study the following drugs were used:
- Dofetilide: a specific I_{kr} blocker used to induce arrhythmias in this model (protocol B&D).
- W-7: a calmodulin inhibitor, and as such an upstream blocker of CaMKII.
- KN93: a direct CaMKII blocker, used in vitro as an acute anti-arrhythmic drug (protocol D)
- Cyclosporin A: 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 Cyclosporin A experiment
Twelve adult mongrel dogs were randomly divided: five received cyclosporin A (CSA, 10 mg/kg/BID) treatment from one week before AV-block until two weeks thereafter (Fig. 1A), while seven others served as a control group. Animals were tested twice: 1) at sinus rhythm and directly after AV-block, and 2) two weeks later (CAVB2). Besides regular measurements, LV pressure (Sentron, Roden, the Netherlands) and $+dP/dT$[11] was determined. At CAVB2 an arrhythmia challenge was performed using dofetilide (25µg/kg/ 5 minutes, or till 1st TdP). Afterwards, dogs were sacrificed to obtain heart weight, biopsies, and cardiomyocytes.
The effectiveness of CSA as a calcineurin inhibitor was confirmed using quantitative PCR on Rcan1-4, a gene downstream of calcineurin (for the protocol see supplemental methods).

Protocol B: W-7 in vivo anti-arrhythmia experiment
At CAVB (≥5 weeks), W-7 (50µmol/kg/5' [12]) (Biomol) was given as an anti-arrhythmic drug (Fig. 2A) against TdPs (N=7). The number of TdPs was quantified per 5 minutes interval. STV was determined over 30 consecutive beats, with the following formula: $STV = \sum 1.30|Dn-Dn-1|/(30 \times \sqrt{2})$, where D represents LV MAPD90[13].

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 Q-PCR. For the complete protocol see supplemental methods.

Protocol D: Patch clamp on cardiomyocytes
Hearts were excised and cardiomyocytes enzymatically dissociated, as described before[14]. 19 cells from 6 dogs from protocol B were used, of which 9 were treated with KN-93, 6 with W-7, and 4 with cyclosporin. Cells were patch clamped and the experiments conducted (Fig. 4A and supplemental methods).

Protocol E: $[Ca^{2+}]_i$ during dofetilide treatment
In cardiomyocytes (N=13 from 6 dogs of protocol B), calcium transients were determined at baseline and with dofetilide (see Fig. 5A and B for examples). For the full protocol, see supplemental methods.

Protocol F: Acute treatment of cardiomyocytes with dofetilide and W-7
Cardiomyocytes (protocol B) were transfered to culture dishes (+/- 50% confluency) and incubated at 37ºC for 10 minutes in Normal Tyrode solution with 1µM dofetilide, or 1µM dofetilide + 50µM W-7, or 1µM isoproterenol (Fig. 5F). After incubation cells were lysed and frozen. CaMKII autophosphorylation levels was determined via western blotting. (see protocol C).

Statistics
All data are presented as mean±SD. Tests can be found in supplemental methods.

Results:

Part A: Long-term pro-arrhythmic remodeling is not dependent on calcineurin
Treatment with CSA prior to AV-block did increase heart rate (106±21 vs. 149±22 bts/min, $P<0.01$), but no other electrical parameter. Therefore acute AV-block parameters were set at 100%. Chronic blockade of calcineurin did neither prevent electrical (QTc, Fig 1B) and mechanical remodeling (LV dP/dT, Fig 1C), nor influenced heart weight (9g/kg).

Arrhythmogenicity after dofetilide challenge also remained similar (TdP incidence 5/7 vs. 4/5 dogs). Rcan1-4, a downstream target of calcineurin (Fig 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, we also did not observe an increase in Rcan1-4 expression during the remodeling process in untreated CAVB dogs (Fig. 1E), indicating that calcineurin activity did not alter between sinus rhythm, and 2 (CAVB2) or 9+ weeks AV-block (CAVB9+).

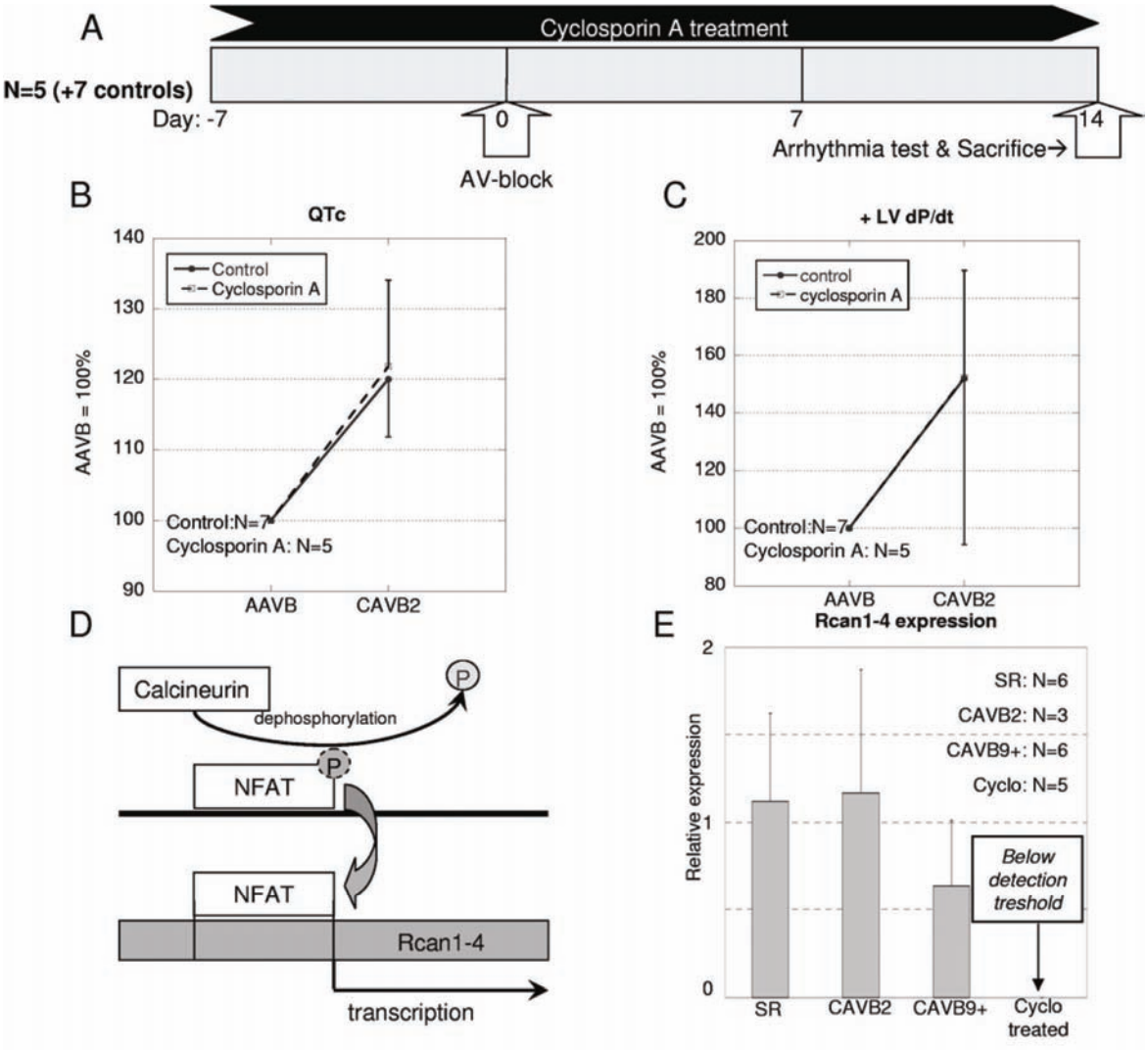
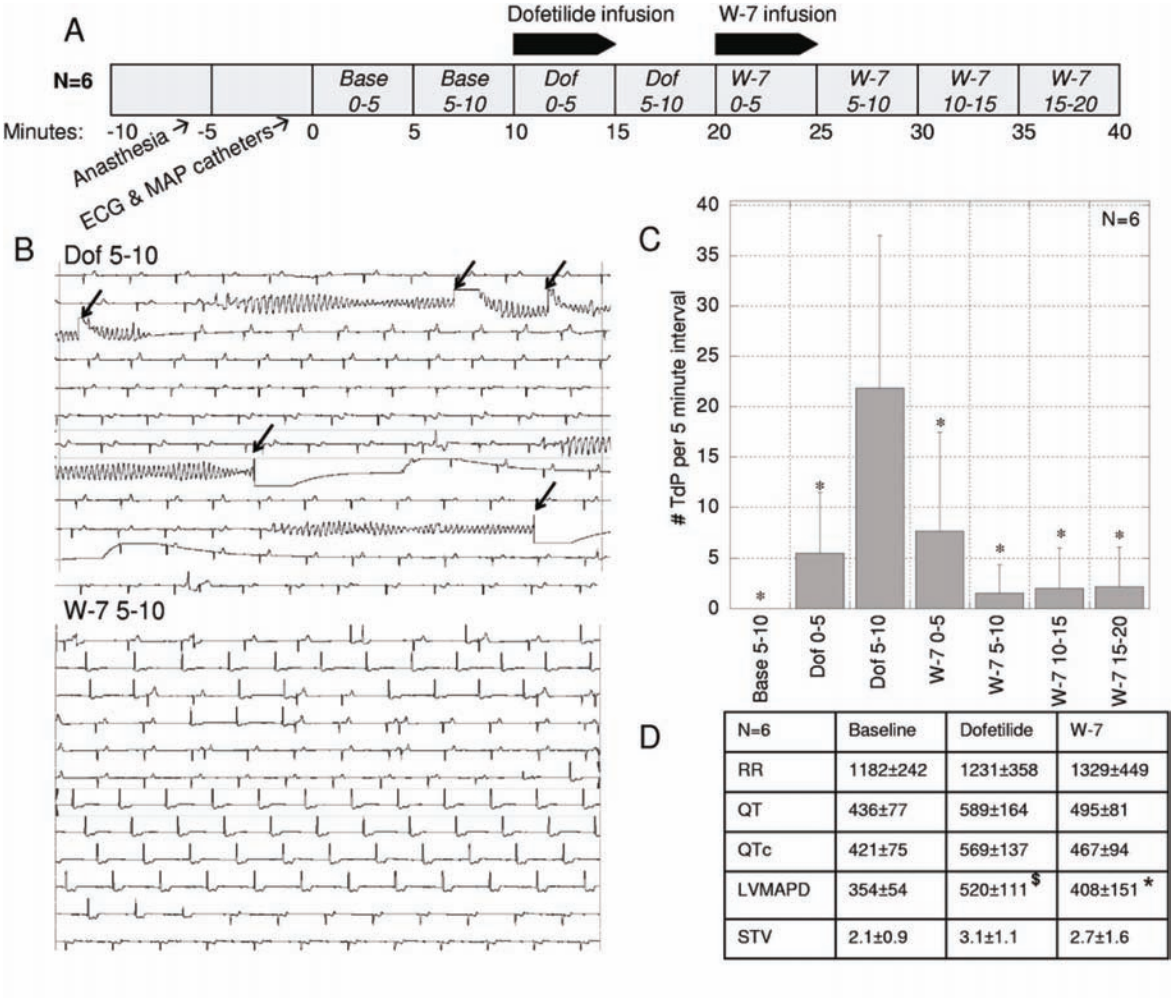


Figure 1:Calcineurin inhibition has no effect on cardiac remodeling and arrhythmogenesis in the CAVB dog
A) Scheme of the in vivo cyclosporin A treatment experiment.
B) Electrical remodeling was quantified as increase in QTc after CAVB with acute AV-block (AAVB) as 100%.
C) Contractile remodeling was quantified as changes in left ventricular dP/dt.
D) Scheme depicting how *regulator of calcineurin 1 exon 4* (RCAN1-4) is linked to calcineurin activation
E) Quantification of Rcan1-4 expression levels

Part B: In vivo CaMKII inhibition is anti-arrhythmic

Dofetilide prolonged QT and induced TdP (Fig. 2B, upper panel). W-7 suppressed almost all TdPs (Fig. 2B, bottom panel). In Fig. 2C the quantification of this effect has been illustrated.

The anti-arrhythmic effect of W-7 was accompanied by LVMAPD shortening (Fig. 2D), but no effect on PP: baseline 582±131 ms, dofetilide 703±225 ms, and after W-7 727±158ms).



N=6	Baseline	Dofetilide	W-7
RR	1182±242	1231±358	1329±449
QT	436±77	589±164	495±81
QTc	421±75	569±137	467±94
LVMAPD	354±54	520±111 $	408±151 *
STV	2.1±0.9	3.1±1.1	2.7±1.6

Figure 2: Calmodulin inhibition is anti-arrhythmic in vivo
A) Schematic overview of the protocol.
B) Examples of ECG tracings during the in vivo dofetilide (upper panel) and W-7 (lower panel) experiments.
C) Quantification of TdP arrhythmias. * = P<0.05 vs. dofetilide infusion
D) Electrophysiological parameters (ms) during the in vivo dofetilide/W-7 experiments. *=P<0.05 compared to dofetilide. $=P<0.05 compared to baseline

Part C: The CaMKII pathway is chronically activated in the CAVB dog, but truncated at HDAC4

Total CaMKII levels (CaMKII-T), did not alter during CAVB (from 1±0.1 at SR, to 0.8±1 at CAVB2, to 1±0.2 at CAVB9+), but autophosphorylation was increased (Fig 3A). Phospholamban, a direct target of CaMKII at Thr17 was also more phosphorylated (Fig 3B). Next we assessed the phosphorylation status of HDAC4, which is a known target of CaMKII and involved in activating a MEF2C-mediated maladaptive gene expression profile[15-17]. In our model of compensated hypertrophy, phosphorylation of HDAC4 was completely identical between SR and CAVB9 (Fig 3C). In contrast, a marker of physiological hypertrophy, Akt [18], was increased (Fig 3D).

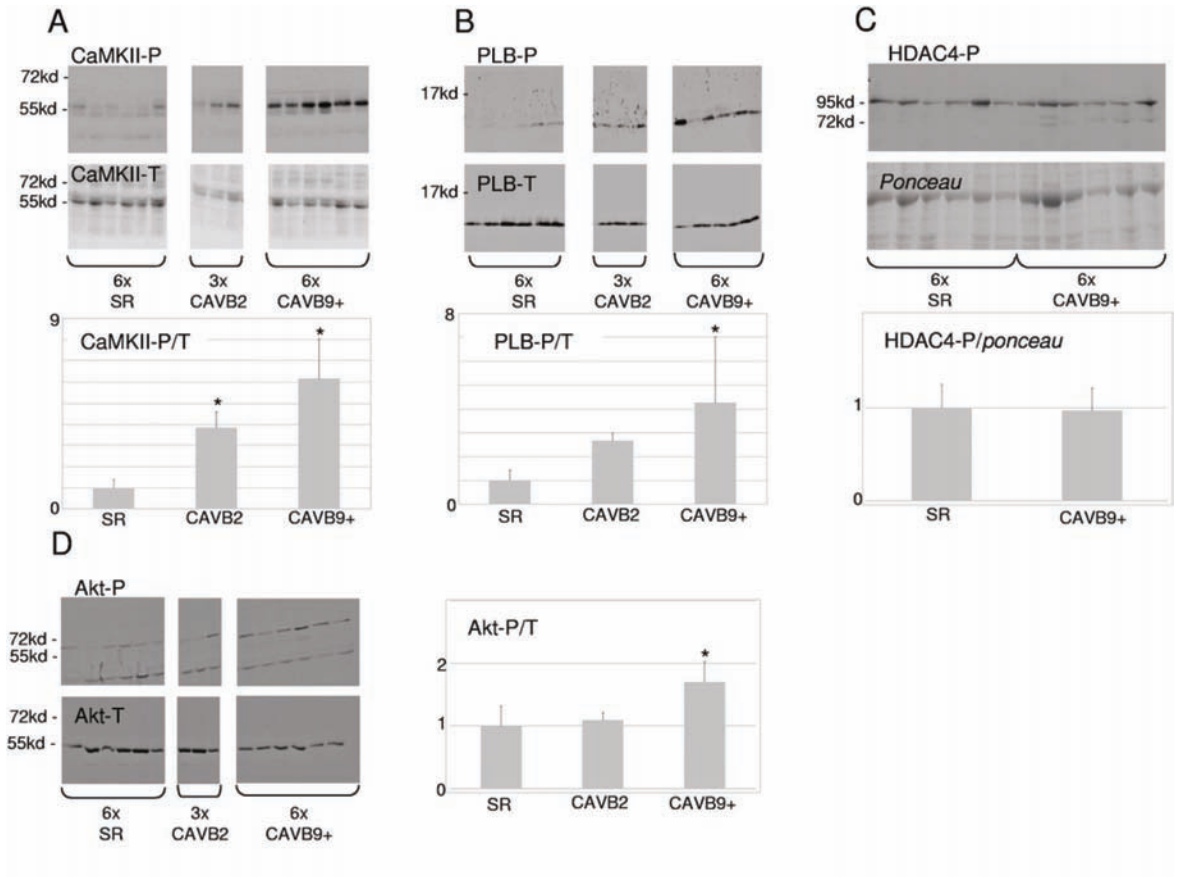


Figure 3: Phosphorylation levels of CaMKII, phospholamban, HDAC4, and Akt during cardiac remodeling in the CAVB dog
A) Representative western blots of phosphorylated CaMKII (CaMKII-P) and total CaMKII (CaMKII-T). * = P<0.05 compared to Sinus rhythm.
B) Idem, but now on phosphorylated phospholamban (PLB-P) 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).

Part D: In vitro CaMKII inhibition is anti-arrhythmic, but calcineurin inhibition is not
Electrically stimulated and dofetilide-treated cardiomyocytes showed AP prolongation (Fig. 4B), increased STV (Fig. 4C), and EADs (Fig. 4D). Once W-7 was co-transfused, EADs disappeared (in 6 out of 6 cells) and APD and STV significantly decreased (Fig. 4B and C). KN-93 showed comparable results (EADs disappeared in 8/9 cells), whereas calcineurin inhibition by CSA was not anti-arrhythmic (Fig. 4D). Results for every individual cell can be found in supplemental figure 1.

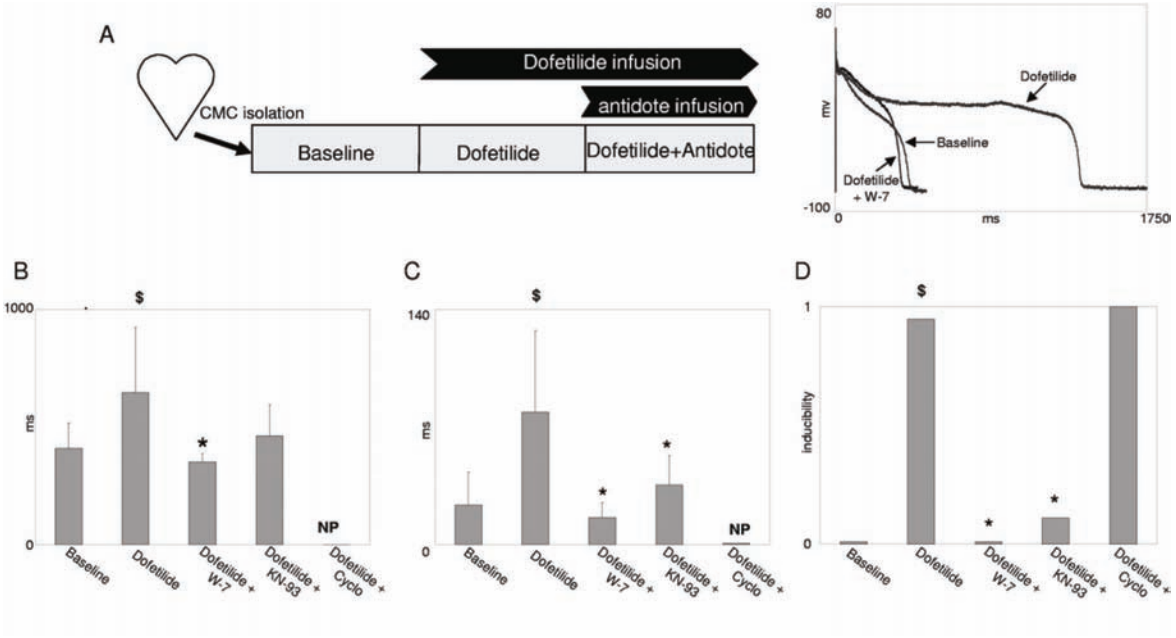


Figure 4: CaMKII inhibition is anti-arrhythmic in vitro, and decreases APD and STV
Quantification of electrical parameters, with the protocol and a representative example in panel **A**. APD90 (**B**) and STV (**C**) were determined in 10 consecutive beats. EADs were scored in panel **D**. NP = not possible $= P<0.05 baseline vs dofetilide. * = P<0.05 dofetilide vs dofetilide+antidote.

Part E+F: Dofetilide treatment increases the Ca-transient and activates CaMKII: an effect suppressed by W7.

Dofetilide increased calcium in CAVB cardiomyocytes, both at diastole (Fig. 5C),systole, (Fig. 5D), as in the amplitude (Fig. 5E). Dofetilide treatment also increased CaMKII autophosphorylation, comparably to the effect induced by the positive control isoproterenol. Co-treatment with W-7 was able to suppress this enhanced CamKII phosphorylation completely. (Fig. 5G)

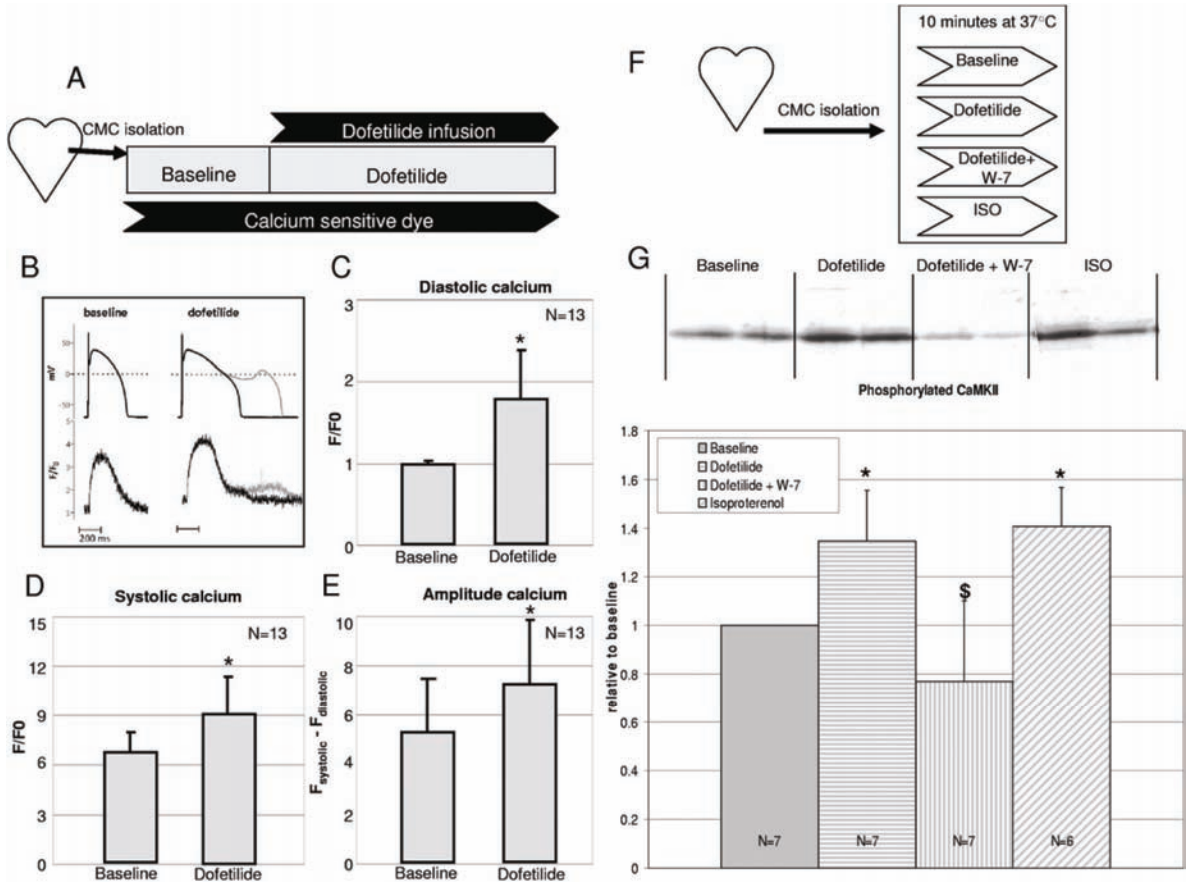


Figure 5: Effects of dofetilide on calcium in CAVB cells.
The protocol as shown in (**A**) was applied. In **B** examples of calcium fluxes are shown. Bar graphs represent quantification of diastolic (**C**), systolic calcium levels (**D**), and calcium amplitude (**E**) in isolated cardiomyocytes at baseline and during dofetilide-infusion. * =P<0.05, compared to baseline.
F) Scheme showing the protocol used to determine the effects of dofetilide on CaMKII in CAVB cells.
G) Representative western blot, and quantification of phosphorylated CaMKII (normalized to total protein levels with ponceau staining) with dofetilide and W-7 treatment, or isoproterenol.
* = P<0.05, compared to baseline. $ = P<0.05, compared to dofetilide.

Discussion:

Cardiac remodeling in the CAVB dog can be sub-divided in a number of processes. Firstly, electrical adaptations, leading to a longer APD, an increased temporal dispersion of repolarization (STV), and increased arrhythmogeneity.[19]. Secondly, cardiac output is preserved by compensatory contractile remodeling [20], and thirdly, biventricular hypertrophy develops [19]. Previously, we demonstrated that structural remodeling did not participate dominantly, because in the absence of hypertrophy, TdP could be induced[11].
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 where CaMKII negatively controls the downstream activity of CnA[21], to a cross-talk, in which both contribute to electrical remodeling[22]. It is even postulated that CaMKII is downstream of CnA, as long-term CaMKII inhibition reduced arrhythmogenesis and improved contractile function in CnA overexpressing mice.[23]

In this model of compensated hypertrophy with a high susceptibility for TdP, we demonstrate that CSA treatment could neither prevent ventricular remodeling nor arrhythmogenesis, nor did CAVB induce activation of CnA, since Rcan1-4 expression remained unaltered (Fig. 1). Chronically, there is an increased phosphorylation of CaMKII and its target phospholamban. HDAC4 phosphorylation remained, however, unchanged while phosphorylation of Akt was increased (Fig. 3). Dofetilide administration increased Ca^{2+} transients (Fig. 5), CaMKII activity (Fig. 5), APD, STV, and induced EADs (Fig. 4). In vivo, dofetilide repeatedly induced TdP (Fig. 2), which could be antagonized by W-7. KN-93 and W-7, but not CSA, were also anti-arrhythmic in vitro (Fig. 4). Thus, using in vivo and in vitro experiments, we conclude that: 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 was able to initiate arrhythmias. The role of CaMKII and CnA in the CAVB dog has been summarized in figure 6.

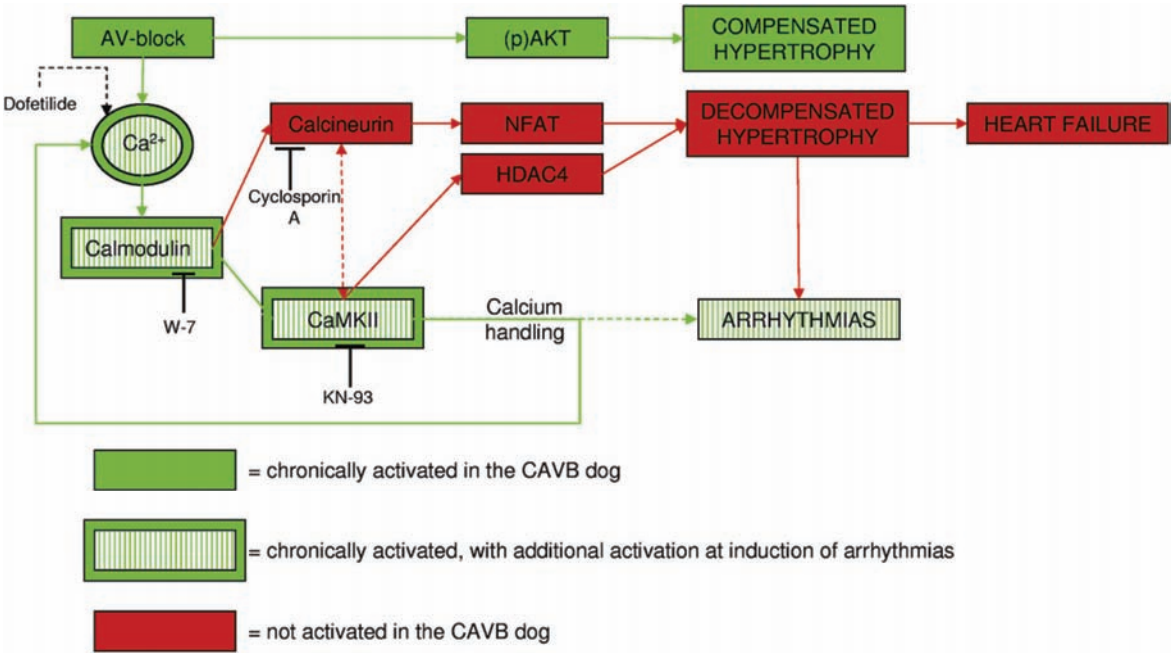


Figure 6: Intracellular signaling pathways involved in hypertrophy & arrhythmias in the CAVB dog
Summary of the observed role of the calcineurin and CaMKII pathways in the CAVB dog as described in this paper.

CnA activity in the CAVB dog

The CnA pathway has been implicated in development of pathological hypertrophy, but not in exercise induced physiological (compensated) hypertrophy[7]. Here we show that remodeling upon AV-block cannot be prevented by CnA inhibtion, and also does not result in increased expression levels of Rcan 1-4, a target of CnA[7]. Moreover, we demonstrate that CSA cannot suppress EADs in vitro. These results are in contrast with a study of Schreiner *et al.*[24] where CSA pre-treatment appeared effective in diminishing hypertrophy in CAVB dogs. In their control group, however, a 43% mortality rate was noticed, suggesting that heart failure may be part of the remodeling. In our hands, mortality after creation of AV-block is less than 5% [25].
Absence of involvement of CnA is further stressed by the notion that activation of the pathway causes re-expression of the voltage gated T-type Ca^{2+}-channel[26]. This channel is, however, not detectable in our CAVB dog model[27]. From our current data with respect to a potential role of CnA in remodeling of the CAVB heart we conclude that both with respect to electrophysiological and structural remodeling, CnA is not involved.

CaMKII activity and compensated hypertrophy in the CAVB dog

Previous studies have shown that chronic activation of CaMKII by, 1) constitutive overexpression in mice[6], or 2) increased expression in failing human myocardium[1, 2] and 3) animal models[8], will eventually result in heart failure, dilatation, arrhythmogenesis and sudden death. More recently, Ling et al.[8] concluded that chronic CaMKII activation and altered gene expression could be restricted to dilated cardiomyopathy in which CaMKII activation leads to a number of adaptations, including altered Ca^{2+}-handling[28], more apoptosis[29], and fibrosis[30].

That the calmodulin/CaMKII pathway is chronically activated in the CAVB dog seems contradictive in this respect, since the compensated hypertrophy in the CAVB dog does not deteriorate into heart failure. One explanation is the intensity of the CaMKII signaling. In the CAVB dog only activity is increased, while in heart failure models both activity *and expression* are higher.

Importantly, in the CAVB model the increased activation without increased expression of CaMKII did not result in higher HDAC4 phosphorylation. Activation of HDAC4 leads to MEF2-induced expression of pathological hypertrophy genes, like ANP, BNP, and β-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 are back to baseline[19], while CaMKII is still chronically activated (shown in this study). Chronic inhibition of CaMKII is known to inhibit deterioration into heart failure, but not hypertrophy as such[8]. Apparently, the signaling downstream of CaMKII differs between compensated and decompensated, and as such, 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 (Fig 3D), which is linked to physiological 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 implies that a previous 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, while the diastolic Ca^{2+} concentration is equal to normal physiologic states as seen in sinus rhythm dogs [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 back to baseline at 5 weeks of remodeling[32], but aldosterone levels, also identified as a source for 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 anti-arrhythmic effect of the calmoduling 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 $[Ca2+]_i$ (Fig. 5).

Potential mechanisms underlying pro-arrhythmic CaMKII activity

The dofetilide-induced CaMKII activation is modest (Fig. 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, the dofetelide-induced overactivation of CaMKII brings the CAVB dog closer to the rabbit model.

Since dofetilide increased CaMKII activity and associated arrhythmias within minutes in our experiments, and its inhibition is anti-arrhythmic within minutes as well, the logical targets might be directly modulated by CaMKII.

A previous study in the CAVB model has learned that triggered activity but not re-entry is the leading cause for arrhythmias in this model[37]. This leaves a limited number of remaining possibilities, namely the modulatory effect on: potassium, sodium-late, and calcium currents. Thus a decrease in the APD/QT or control of $[Ca]_i$ are the key anti-arrhythmic actions. Blockade of CaMKII will decrease activity of potassium channels[10], which is opposite of being anti-arrhythmic, whereas under these conditions both the CaMKII effect on increasing sodium late [38] or the L-type calcium channel is anti-arrhythmic in the CAVB dog[39]. Secondly, a window current exists in CAVB cardiomyocytes, with altered inactivation[27], which is in concordance with changes observed in the CAVB rabbit, where altered L-type calcium channel inactivation could be prevented by CaMKII inhibition[40].

It is difficult to separate effects on APD from that on $[Ca^{2+}]$ handling, since they affect each other. In our experiments, W-7 shortened the APD, questioning a dominant effect on $[Ca^{2+}]$ handling. This in contrast to others whom have shown in a rabbit model of long-QT that W-7 can reduce AP variability and arrhythmias without APD shortening. The latter is a strong suggestion of involvement of CaMKII inhibition [40]. To unravel the exact mechanisms how CaMKII affects ion currents land Ca^{2+}-handling, future studies are required.

Study limitations:

Because of limited KN-93 availibility it was only used 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 as a calmodulin inhibitor. However, other drugs specifically inhibiting CaMKII are still not available, and peptides like AIP, although specific, are not useful for suppression experiments. Similarly, CSA, the well-known inhibitor of CnA exerts effects on other targets too. For that reason, although our data show that CnA is not activated, we can not exclude that other CSA sensitive and unidentified targets have contributed to the effects observed after the intervention with CSA.

In 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 pharmacological challenge initiated arrhythmias based on triggered activity.

Reference List

1. Hoch B, Meyer R, Hetzer R, Krause EG, Karczewski P. Identification and expression of delta-isoforms of the multifunctional Ca2+/calmodulin-dependent protein kinase in failing and nonfailing human myocardium. Circ Res 1999 Apr 2; 84(6): 713-21.

2. Kirchhefer U, Schmitz W, Scholz H, Neumann J. Activity of cAMP-dependent protein kinase and Ca2+/calmodulin-dependent protein kinase in failing and nonfailing human hearts. Cardiovasc Res 1999 Apr; 42(1): 254-61.

3. Lim HW, Molkentin JD. Calcineurin and human heart failure. Nat Med 1999 Mar; 5(3): 246-7.

4. Molkentin JD, Lu JR, Antos CL, et al. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 1998 Apr 17; 93(2): 215-28.

5. Wu Y, Temple J, Zhang R, et al. Calmodulin kinase II and arrhythmias in a mouse model of cardiac hypertrophy. Circulation 2002 Sep 3; 106(10): 1288-93.

6. Maier LS, Zhang T, Chen L, DeSantiago J, Brown JH, Bers DM. Transgenic CaMKIIdeltaC overexpression uniquely alters cardiac myocyte Ca2+ handling: reduced SR Ca2+ load and activated SR Ca2+ release. Circ Res 2003 May 2; 92(8): 904-11.

7. Bourajjaj M, Armand AS, da Costa Martins PA, et al. NFATc2 is a necessary mediator of calcineurin-dependent cardiac hypertrophy and heart failure. J Biol Chem 2008 Aug 8; 283(32): 22295-303.

8. Ling H, Zhang T, Pereira L, et al. Requirement for Ca2+/calmodulin-dependent kinase II in the transition from pressure overload-induced cardiac hypertrophy to heart failure in mice. J Clin Invest 2009 May; 119(5): 1230-40.

9. Anderson ME, Braun AP, Wu Y, et al. KN-93, an inhibitor of multifunctional Ca++/calmodulin-dependent protein kinase, decreases early afterdepolarizations in rabbit heart. J Pharmacol Exp Ther 1998 Dec; 287(3): 996-1006.

10. Wagner S, Hacker E, Grandi E, et al. Ca/calmodulin kinase II differentially modulates potassium currents. Circ Arrhythm Electrophysiol 2009 Jun; 2(3): 285-94.

11. Schoenmakers M, Ramakers C, van Opstal JM, Leunissen JD, Londono C, Vos MA. Asynchronous development of electrical remodeling and cardiac hypertrophy in the complete AV block dog. Cardiovasc Res 2003 Aug 1; 59(2): 351-9.

12. Mazur A, Roden DM, Anderson ME. Systemic administration of calmodulin antagonist W-7 or protein kinase A inhibitor H-8 prevents torsade de pointes in rabbits. Circulation 1999 Dec 14; 100(24): 2437-42.

13. Thomsen MB, Verduyn SC, Stengl M, et al. Increased short-term variability of repolarization predicts d-sotalol-induced torsades de pointes in dogs. Circulation 2004 Oct 19; 110(16): 2453-9.

14. Nalos L, Varkevisser R, Jonsson M, et al. Comparison of I(Kr) blocking drugs Moxifloxacin and Dofetilide/E-4031 in 5 screening models of pro-arrhythmia reveals insufficient specificity of isolated cardiomyocytes. Br J Pharmacol 2011 Jun 30.

15. Backs J, Song K, Bezprozvannaya S, Chang S, Olson EN. CaM kinase II selectively signals to histone deacetylase 4 during cardiomyocyte hypertrophy. J Clin Invest 2006 Jul; 116(7): 1853-64.

16. Backs J, Backs T, Neef S, et al. The delta isoform of CaM kinase II is required for pathological cardiac hypertrophy and remodeling after pressure overload. Proc Natl Acad Sci U S A 2009 Feb 17; 106(7): 2342-7.

17. Zhang T, Kohlhaas M, Backs J, et al. CaMKIIdelta isoforms differentially affect calcium handling but similarly regulate HDAC/MEF2 transcriptional responses. J Biol Chem 2007 Nov 30; 282(48): 35078-87.

18. DeBosch B, Treskov I, Lupu TS, et al. Akt1 is required for physiological cardiac growth. Circulation 2006 May 2; 113(17): 2097-104.

19. Vos MA, de Groot SH, Verduyn SC, et al. Enhanced susceptibility for acquired torsade de pointes arrhythmias in the dog with chronic, complete AV block is related to cardiac hypertrophy and electrical remodeling. Circulation 1998 Sep 15; 98(11): 1125-35.

20. de Groot SH, Schoenmakers M, Molenschot MM, Leunissen JD, Wellens HJ, Vos MA. Contractile adaptations preserving cardiac output predispose the hypertrophied canine heart to delayed afterdepolarization-dependent ventricular arrhythmias. Circulation 2000 Oct 24; 102(17): 2145-51.

21. MacDonnell SM, Weisser-Thomas J, Kubo H, et al. CaMKII negatively regulates calcineurin-NFAT signaling in cardiac myocytes. Circ Res 2009 Aug 14; 105(4): 316-25.

22. Xiao L, Coutu P, Villeneuve LR, et al. Mechanisms underlying rate-dependent remodeling of transient outward potassium current in canine ventricular myocytes. Circ Res 2008 Sep 26; 103(7): 733-42.

23. Khoo MS, Li J, Singh MV, et al. Death, cardiac dysfunction, and arrhythmias are increased by calmodulin kinase II in calcineurin cardiomyopathy. Circulation 2006 Sep 26; 114(13): 1352-9.

24. Schreiner KD, Kelemen K, Zehelein J, et al. Biventricular hypertrophy in dogs with chronic AV block: effects of cyclosporin A on morphology and electrophysiology. Am J Physiol Heart Circ Physiol 2004 Dec; 287(6): H2891-H2898.

25. Oros A, Beekman JD, Vos MA. The canine model with chronic, complete atrio-ventricular block. Pharmacol Ther 2008 Aug; 119(2): 168-78.

26. Chiang CS, Huang CH, Chieng H, et al. The Ca(v)3.2 T-type Ca(2+) channel is required for pressure overload-induced cardiac hypertrophy in mice. Circ Res 2009 Feb 27; 104(4): 522-30.

27. Antoons G, Volders PG, Stankovicova T, et al. Window Ca2+ current and its modulation by Ca2+ release in hypertrophied cardiac myocytes from dogs with chronic atrioventricular block. J Physiol 2007 Feb 15; 579(Pt 1): 147-60.

28. Ai X, Curran JW, Shannon TR, Bers DM, Pogwizd SM. Ca2+/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca2+ leak in heart failure. Circ Res 2005 Dec 9; 97(12): 1314-22.

29. Palomeque J, Rueda OV, Sapia L, et al. Angiotensin II-induced oxidative stress resets the Ca2+ dependence of Ca2+-calmodulin protein kinase II and promotes a death pathway conserved across different species. Circ Res 2009 Dec 4; 105(12): 1204-12.

30. Zhang W, Chen DQ, Qi F, Wang J, Xiao WY, Zhu WZ. Inhibition of calcium-calmodulin-dependent kinase II suppresses cardiac fibroblast proliferation and extracellular matrix secretion. J Cardiovasc Pharmacol 2010 Jan; 55(1): 96-105.

31. Sipido KR, Volders PG, de Groot SH, et al. Enhanced Ca(2+) release and Na/Ca exchange activity in hypertrophied canine ventricular myocytes: potential link between contractile adaptation and arrhythmogenesis. Circulation 2000 Oct 24; 102(17): 2137-44.

32. Volders PG, Sipido KR, Vos MA, Kulcsar A, Verduyn SC, Wellens HJ. Cellular basis of biventricular hypertrophy and arrhythmogenesis in dogs with chronic complete atrioventricular block and acquired torsade de pointes. Circulation 1998 Sep 15; 98(11): 1136-47.

33. He BJ, Joiner ML, Singh MV, et al. Oxidation of CaMKII determines the cardiotoxic effects of aldosterone. Nat Med 2011 Dec; 17(12): 1610-8.

34. Erickson JR, Joiner ML, Guan X, et al. A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. Cell 2008 May 2; 133(3): 462-74.

35. Tsuji Y, Opthof T, Yasui K, et al. Ionic mechanisms of acquired QT prolongation and torsades de pointes in rabbits with chronic complete atrioventricular block. Circulation 2002 Oct 8; 106(15): 2012-8.

36. Tsuji Y, Hojo M, Voigt N, et al. Ca(2+)-related signaling and protein phosphorylation abnormalities play central roles in a new experimental model of electrical storm. Circulation 2011 May 24; 123(20): 2192-203.

37. Boulaksil M, Jungschleger JG, Antoons G, et al. Drug-Induced Torsade de Pointes Arrhythmias in the Chronic AV Block Dog Are Perpetuated by Focal Activity. Circ Arrhythm Electrophysiol 2011 Aug 1; 4(4): 566-76.

38. Antoons G, Oros A, Beekman JD, et al. Late na(+) current inhibition by ranolazine reduces torsades de pointes in the chronic atrioventricular block dog model. J Am Coll Cardiol 2010 Feb 23; 55(8): 801-9.

39. Qi X, Yeh YH, Chartier D, et al. The calcium/calmodulin/kinase system and arrhythmogenic afterdepolarizations in bradycardia-related acquired long-QT syndrome. Circ Arrhythm Electrophysiol 2009 Jun; 2(3): 295-304.

40. Qi X, Yeh YH, Chartier D, et al. The calcium/calmodulin/kinase system and arrhythmogenic afterdepolarizations in bradycardia-related acquired long-QT syndrome. Circ Arrhythm Electrophysiol 2009 Jun; 2(3): 295-304.

41. Gao L, Blair LA, Marshall J. CaMKII-independent effects of KN93 and its inactive analog KN92: reversible inhibition of L-type calcium channels. Biochem Biophys Res Commun 2006 Jul 14; 345(4): 1606-10.

Supplemental Methods

Anesthesia, AV-node ablation, and used drugs
Premedication consisted of 0.5mg/kg methadone, 0.5mg/kg acepromazine and 0.02mg/kg atropine i.m.. Anesthesia was induced with pentobarbital (Nembutal 25/mg/kg i.v.) or thiopental (15/mg/kg) and maintained by isoflurane (1.5% in O_2 and N_2O, 1:2), comparable to [1-3]. Lack of pupillary response was checked to assure adequate anesthesia. In addition, heart and respiratory rate were constantly monitored. AV-block was induced via radiofrequency ablation, which led to an acute drop in heart rate from 117±28 to 58±14 beats per minute. During all in vivo experiments, a six lead ECG and two monophasic action potentials (MAP) (EP Technologies, Sunnyvale, CA, USA) were recorded from the endocardium both in the left (LV) and right ventricle (RV).
A 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 sec), the animals were defibrillated through patches placed in advance.

Protocol C: Ventricular biopsies
During open-chest surgery, three simultaneous LV free wall transmural biopsies were taken using a 14G automatic biopsy needle (Acecut, TSK laboratory, Japan), and subsequently snap frozen in liquid nitrogen and stored at -80ºC. Biopsies were taken serially either at sinus rhythm and 2 weeks AV-block (3 dogs), or at sinus rhythm and 9+ weeks AV-block (3 dogs), to isolate protein and/or RNA (TRIzol reagent) for subsequent Western blots or Q-PCR. In an additional three animals, biopsies were taken only at 9+ weeks AV-block.

Q-PCR with Rcan1-4 primers
Expression levels of Rcan1-4 [1] were determined by quantitative PCR (Bio-rad iCycler machine), using iQ™ SYBR® Green Supermix (Biorad).
GAPDH was used for normalisation. For GAPDH the following primers were used: forward - 5'-AGTCAAGGCTGAGAACGGAAACT-3', and reverse - 5'-TCCACAACATACTCAGCACCAGCA-3'. The Rcan1-4 primers were as follows: forward - 5'-ACCGCTTAGCGCTTTCACTG-3', and reverse - 5'-TGTTTGCCACACACAGGCAATC-3'.

Western blots
Biopsies were pulverized using a pre-cooled 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-CaMKIIδ (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).

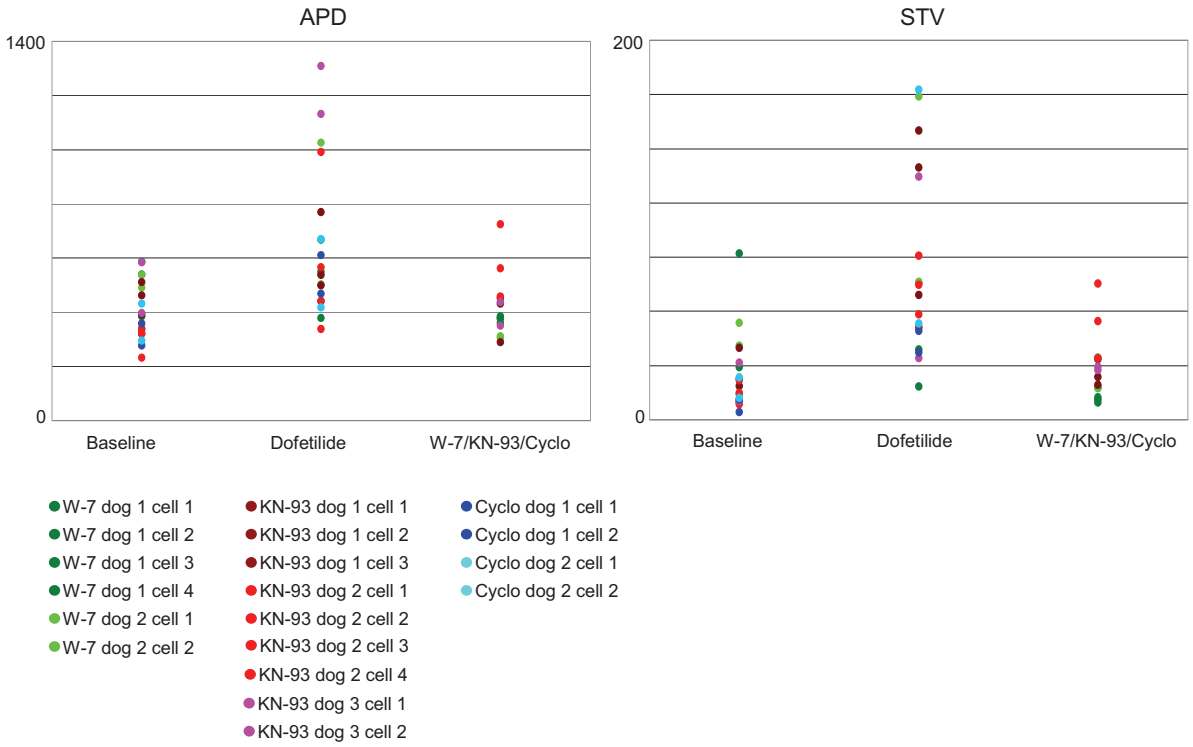
Protocol D: Patch clamp experiments on left ventricular isolated cardiomyocytes
Hearts were quickly excised and single cardiomyocytes were enzymatically dissociated from the LV midmyocardial layer, stored in Normal Tyrode solution, as described before[2]. Cells were placed in a perfusion bath and all experiments were performed at 37ºC. 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 afterdepolarization. Experiments were performed in Normal Tyrode solution containing (in mmol/L): 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 11.8 HEPES and 10 glucose, pH 7.4. Pipettes had a resistance of 2-3 MΩ when filled with pipette solution, containing (in mmol/L): 130 KCl, 10 NaCl, 10 HEPES, 5 MgATP and 0.5 $MgCl_2$, pH 7.2.
Cells were superfused with Normal Tyrode solution and after baseline recording dofetilide (1μM) was added to provoke EADs. After the induction of at least three EADs by dofetilide (1μM [3]), an antidote was added (see Fig. 4A): W-7 (50μM, Biomol), CSA (1 mg/ml[4, 5], Neoral) or KN-93 (Sigma, 1 μM, [6]).

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 Fig. 5A, and B for examples), using a Zeiss Axiovert 100M inverted microscope with a x40/1.3 oil-immersion objective and a Zeiss LSM 510 confocal laser point-scanning system (Zeiss GmbH). Fluo 3 and di-8-ANEPPS were excited with the 488-nm line of a 25-mW argon laser. To record $[Ca^{2+}]_i$ transients, cells were scanned along the longitudinal axis, orthogonal to the Z lines, avoiding scanning through nuclei. [7]

Statistics
The following tests have been performed:
- Unpaired t-test: Figure 1B-C, 3C, and 5C-E.
- Fisher's Exact test: Figure 4D
- ANOVA followed by a Holm-Sidak post-hoc test: Figure 1E, 3A, B, D, and figure 5G.
- Repeated measures ANOVA followed by a Holm-Sidak post-hoc test: Figure 2C, D, and figure 4B, C. In figure 2C the comparison in the post-hoc test was against the dofetilide group.

Supplemental figure 1



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: W7 in shades of green, KN 93 in brown-red-pink and Cyclosporin 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 were not possible to obtain due to the persistence of EADs.

Reference List

1 Bourajjaj M, Armand AS, da Costa Martins PA et al. NFATc2 is a necessary mediator of calcineurin-dependent cardiac hypertrophy and heart failure. *J Biol Chem* 2008 August 8;283(32):22295-303.

2 Nalos L, Varkevisser R, Jonsson M et al. Comparison of I(Kr) blocking drugs Moxifloxacin and Dofetilide/E-4031 in 5 screening models of pro-arrhythmia reveals insufficient specificity of isolated cardiomyocytes. *Br J Pharmacol* 2011 June 30.

3 Antoons G, Oros A, Beekman JD et al. Late na(+) current inhibition by ranolazine reduces torsades de pointes in the chronic atrioventricular block dog model. *J Am Coll Cardiol* 2010 February 23;55(8):801-9.

4 Taigen T, De Windt LJ, Lim HW, Molkentin JD. Targeted inhibition of calcineurin prevents agonist-induced cardiomyocyte hypertrophy. *Proc Natl Acad Sci U S A* 2000 February 1;97(3):1196-201.

5 Molkentin JD, Lu JR, Antos CL et al. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* 1998 April 17;93(2):215-28.

6 Anderson ME, Braun AP, Wu Y et al. KN-93, an inhibitor of multifunctional Ca++/calmodulin-dependent protein kinase, decreases early afterdepolarizations in rabbit heart. *J Pharmacol Exp Ther* 1998 December;287(3):996-1006.

7 Heinzel FR, Bito V, Volders PG, Antoons G, Mubagwa K, Sipido KR. Spatial and temporal inhomogeneities during Ca2+ release from the sarcoplasmic reticulum in pig ventricular myocytes. *Circ Res* 2002 November 29;91(11):1023-30.

Chapter 5:

Chronic CaMKII inhibition in the AC3-I mouse model positively influences conduction velocity but is not anti-arrhythmic

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In preparation

Abstract

Introduction:
CaMKII activity is enhanced in (progression to) heart failure and appears to be involved in arrhythmogenesis. Suppressing CaMKII activity by AC3-I has been shown to reduce afterdepolarizations in vitro, as well as PES induced arrhythmias in CNA overexpressing mice. To confirm the arrhythmic role of CaMKII, we have tested arrhythmic susceptibility of these AC3-I hearts after application of pressure overload.

Methods:
AC3-I SHAM (N=11) or *transverse aortic constriction* (TAC, N=11) operated mice were compared to similar mice without CaMKII inhibition (historical data). 16 weeks post-surgery, ECG and echo was performed in vivo. Conduction velocity and arrhythmogeneity were determined on Langendorfff perfused hearts via epicardial mapping using an electrode grid. Tachycardias were induced by pacing. Molecular characteristics were determined on the hearts after the Langendorfff experiments.

Results:
Echo showed an aortic pressure gradient of 63±16% in the TAC group. The hearts were dilated (LV inner diameter, SHAM: 3.2±0.05 and TAC: 3.8±0.06mm*) and contractile performance was decreased (Fractional shortening, SHAM: 48.4±2.6 and TAC: 35.8±5.5 %*). Heart weight & lung weight to tibia length showed higher values in the TAC group. Heart rate was not different, but the QRS interval was slightly longer in the TAC group (11.47±2.7 vs. 9.05±2.4ms* vs. SHAM).
SHAM mice showed no arrhythmias, while in 36% of the TAC hearts arrhythmias could be induced, which was comparable to TAC mice without CaMKII inhibition: Boulaksil et al., Eur J Heart Failure 2011: 8/18 = 44%[1], Jansen et al., Heart Rhythm 2012: 2/11 = 18%[2]. In contrast to previous studies, TAC did not reduce conduction velocity, and chronic CaMKII inhibition even increased conduction in the SHAM group as compared to WT.
Molecularly, this was accompanied with a preservation of Cx43 and $Na_v1.5$ expression. Fibrosis, however, was markedly increased after TAC.

Conclusion:
After 16 weeks of TAC, chronic CaMKII inhibition preserved conduction velocity and associated molecular parameters. However, it did neither prevent development of hypertrophy and impaired cardiac function, nor the incidence of ventricular tachycardias.

Abbreviations:

AC3-I: autocamtide-3 related peptide
CaMKII: calcium/calmodulin-dependent protein kinase II
CNA: calcineurin A
CV: conduction velocity
Cx: connexin
FS: fractional shortening
HW/BW/TL: heirt weight / body weight / tibia length
LV: left ventricle
Nav1.5: voltage-gated sodium channel, type V
Ncad: N-cadherin
PES: programmed electrical stimulation
RV: right ventricle
TAC: transverse aortic constriction
WT: wild type

Introduction

It has been well established that CaMKII is involved in cardiac arrhythmias, both in models with pharmaceutically induced arrhythmias [3], and in models with chronic CaMKII activation [4] [5]. In general, the available evidence suggests that CaMKII activation can lead to arrhythmias via three potential mechanisms. Firstly, it can directly phosphorylate ion channels, thereby altering their function in a pro-arrhythmic manner [6]. Secondly, prolonged CaMKII activation gives rise to changes in ion channel expression patterns, and as such induces a potential pro-arrhythmic electrical remodeling [7]. Thirdly, long-term CaMKII activation ultimately culminates in cardiac hypertrophy and heart failure [8], and is also involved in cardiomyocyte apoptosis and fibrosis formation [9]. In such a setting re-entry dependent arrhythmias are prone to develop [10].
An arrhythmic model with severe cardiac remodeling is the mouse model of pressure overload due to transverse aortic constriction.[11] TAC surgery in mice leads to hypertrophy with reduced fractional shortening, as well as reduced conduction velocity and ventricular arrhythmias, with the suggestion of an underlying re-entry based mechanism, at least in the perpetuation of arrhythmias [12]. Moreover, it leads to elevated CaMKII expression-, and activation levels [13].
Previously, our group has deliberately investigated the factors involved in the slowed conduction velocity in the mouse heart, and has identified disturbed Cx43[14, 15] and Nav1.5[2] expression patterns, as well as increased cardiac fibrosis[16]. To result in arrhythmias, a combined deterioration of these factors is probably necessary to decrease conduction velocity and a disturbance in only one factor does not seem to be sufficient[17, 18].
A number of studies have investigated the role of CaMKII in TAC mice hearts. An increased expression and activity was observed, and inhibition seemed to prevent a deterioration of fractional shortening[9, 19, 20]. In stead of looking at electrical parameters and arrhythmias however, they have focused mainly on cardiac function[9, 19, 21]. In this study we evaluated the effect of chronic CaMKII inhibition on conduction velocity and arrhythmias.

Materials and Methods

Mice

Mice with cardiac specific expression of autocamtide-3 related peptide (AC3-I) were kindly provided by the lab of Dr. Anderson (The University of Iowa, USA). AC3-I is an effective CaMKII inhibitor (see also supplemental figure 1). The mice were bred in a C57BL/6 background. For the experiments, 3 months old mice were used of either sex. All experiments were approved by the institutional ethical committee for animal experiments of the UMC Utrecht.

Experimental set-up

The experiment started with constriction of the aorta (TAC) (N=11), or sham surgery (SHAM) for the control group (N=11). After 16 weeks subsequent measurements were performed, consisting of cardiac echo and ECG under anesthesia *in vivo*, epicardial mapping *ex vivo*, and histology *in vitro* (Fig 1A).

Aortic constriction

Mice were anesthetized by isoflurane (1.5%, in oxygen), intubated with a polyethylene catheter and ventilated with a rodent ventilator (Minivent, Hugo Sachs Electronics, Germany). A small incision in the second intercostal space was used to reach the aorta. Constriction was

performed by tying a silk suture around the aorta and a 27-gauge needle. The needle was subsequently removed. The tying procedure never took more than 12 seconds. SHAM animals received the same treatment, but without constriction. Effective constriction was confirmed by Doppler echocardiography (pressure gradient: 63±16mmHg). Procedures were conducted as previously described [14].

***In vivo*: Echo and ECG**

In anesthetized mice (1.5% isoflurane), a 3 lead ECG was recorded on a custom built ECG-amplifier (see: [15]) and analyzed off-line using Chart 5 Pro (AD Instruments).
Echocardiography was performed immediately after ECG analysis, to determine functional and structural characteristics (digital cardiac ultrasound platform, SONOS 5500, Philips Medical Systems).

***Ex vivo*: Langendorff perfusion and epicardial mapping**

After the in vivo measurements, the heart was excised and connected to a Langendorff perfusion set-up (Fig 1B, left panel). Perfussion buffer was carbogen-gassed and kept at 37ºC (buffer: NaCl 226, KCl 5, $MgSO_4$ 1.1, NaH_2PO_4 0.35, Na HCO_3 27, glucose 10, mannitol 16 and CaCl2 1.8, all in mmol/L). Additional details can be found in: [15]
A multielectrode 16x13 grid (Fig 1B, left panel) was placed on the epicardial surface of the heart. Stimulation was performed from the center of the grid (2x stimulation threshold).
To assess arrhythmias, a three step protocol was performed:

1) Assess spontaneous arrhythmias.
2) Administer a 16-paced train (cycle length 100ms), followed by one to three premature stimuli close to the effective refractory period.
3) Approximately 2 second burst pacing with the shortest possible cycle length.

Analysis of conduction velocity was done off-line. The maximal negative dV/dt on the unipolar electrogram was defined as the time of activation (Fig 1B, right panel). If in doubt, a laplacian was used to determine whether a signal was local or remote (a laplacian that is first positive, then negative, implies a passing wave front, thus a local signal. Remote signals should almost disappear on a laplacian). The combined activation times of the electrodes allows to generate an activation map, which can be subsequently used to determine conduction velocities (Fig 1B, middle panel).All measurements were determined with custom written software based on Matlab (The MathWorks Inc.), see also: [22]

***In vitro*: Histology and immunohistochemistry**

Hearts were removed from the Langendorff set-up, quickly frozen in liquid nitrogen, and stored at -80ºC. 4 chamber view cryo-sections were generated. Evaluation of Nav1.5 and Cx43 protein expression was performed by immunohistochemistry. Nav1.5 was labeled with a custom-made antibody kindly provided by Dr. Hund (Biomedical Engineering, Columbus, USA), and Cx43 was labeled with a commercially available antibody (Invitrogen, USA).
At least four photos per ventricle were taken for every heart, divided over different sections. Analysis was done in ImageJ and microscopy was performed by a Nikon Eclipse 80i microscope.

Statistics:

All data are expressed as mean±standard deviation. With two groups a student's t-test was performed, with multiple groups an ANOVA using the SigmaStat statistical software package. AC3-I mice data were compared against WT mice from Jansen *et al*..[2] Conduction velocity measurements were repeated to exclude interpersonal variation.

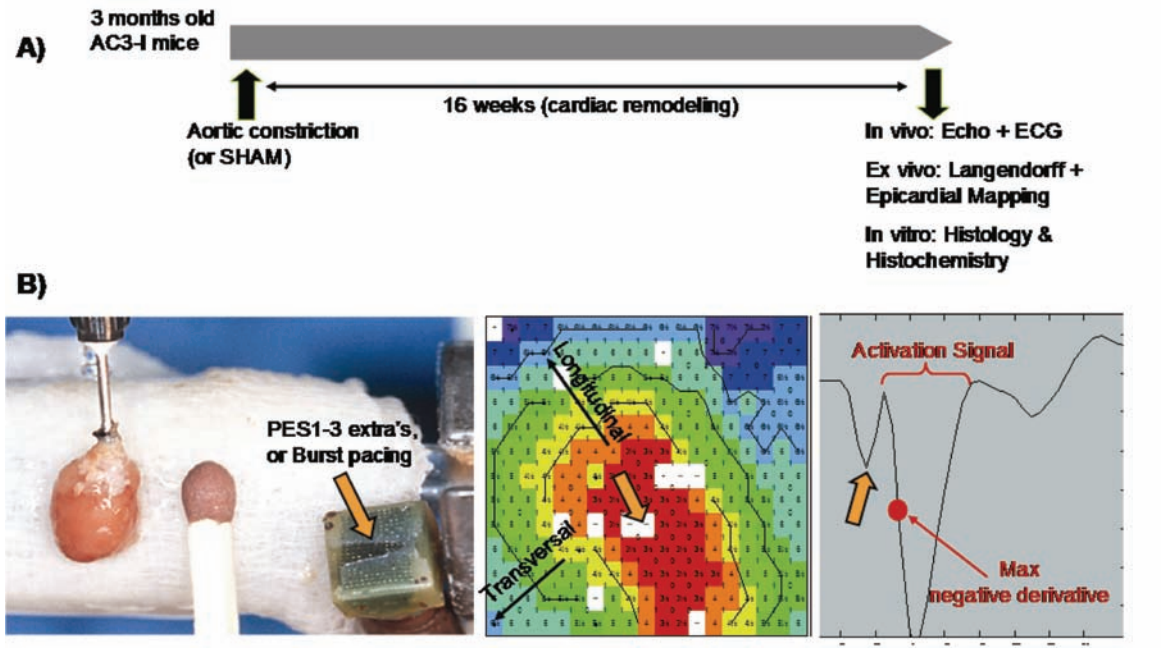


Figure 1: Study set-up and epicardial mapping

A) An overview of the study set-up. First surgery (aortic banding or SHAM) was performed, then a 16 week interval was preserved for cardiac remodeling, followed by the final experiments, where the echo and ECG measurements were done *in vivo*, afterwards the epicardial mapping was performed in an *ex vivo* Langendorff-perfusion set up.

B) Depiction of the epicardial mapping experiments. In the first picture a Langendorff-perfuced mouse heart is shown on the left, and on the right the electrode grid is shown (electrodes are visible as tiny shining dots). The grid was placed on the epicardial surface of the heart. Pacing (orange arrow) was performed from a central electrode. The middle picture is an example of an epicardial map. From the pacing site in the middle the electrical activation occurs, first in the centre (red squares), and finally traversing to the periphery (yellow-green-blue). Longitudinal conduction proceeds more rapidly than transversal conduction, which is perpendicular to fiber orientation. Every square represents one electrode. The last picture explains how we determined the activation at an electrode. The electrode first registers the pacing spike, followed by the activation signal. The maximum negative derivative was considered to be local activation.

Results

Mice with cardiac specific AC3-I expression are not protected against TAC-induced cardiac remodeling

Heart weight measurements (corrected for body weight or tibia length) confirmed cardiac hypertrophy in AC3I mice after 16 weeks of aortic constriction. This effect was comparable to a TAC-induced increase in heart weight in WT mice (Fig 2A). In a comparable fashion, cardiac function deteriorated, defined as significantly reduced fractional shortening as determined via echocardiography in the TAC group versus the SHAM group (Fig 2B). Signs of dilatation were also found, as observed in increased lumen diameters, for example in the LV at diastole (Fig 2C). In addition, wall thickness was increased (Fig 2D).

Reduced cardiac function was accompanied by increased lung weight, a marker for left ventricular failure, in the TAC group, although corrected for tibia length this reached only borderline significance (P=0.07) (Table 1). Heart weight remained significantly elevated, even when normalized for tibia length (Table 1).
Note that the hypertrophy, increased lumen diameters and wall thickness, as well as the reduced fractional shortening after TAC, were similar as observed in WT mice (Fig 2).

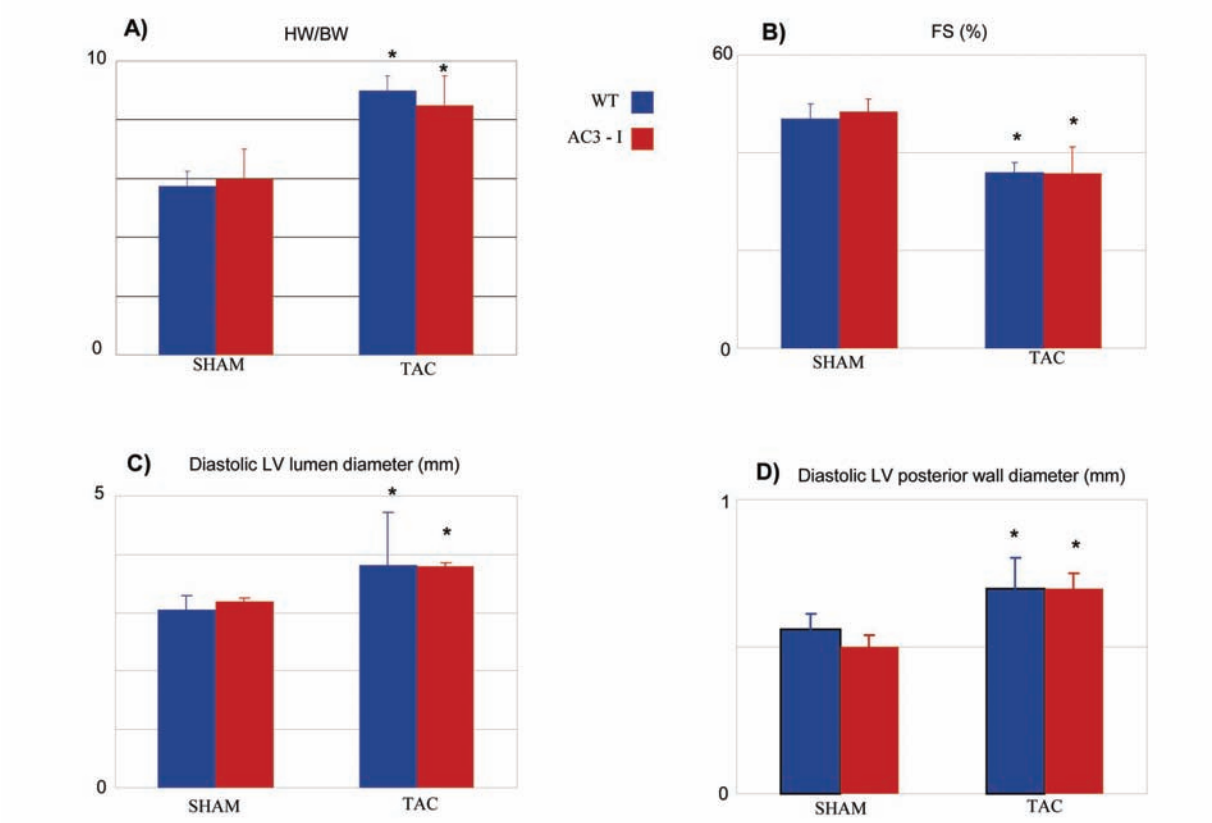


Fig 2: CaMKII inhibition does not prevent cardiac remodeling after aortic constriction

- **A)** Heart weight to body weight in SHAM and TAC groups, in WT and AC3-I mice. *=P<0.05 compared to SHAM
- **B)** Fractional shortening in SHAM and TAC groups, in WT and AC3-I mice. *=P<0.05 compared to SHAM
- **C)** Left ventricular lumen diameter at diastole, in WT and AC3-I mice. *=P<0.05 compared to SHAM
- **D)** Left ventricular posterior wall diameter at diastole, in WT and AC3-I mice. *=P<0.05 compared to SHAM

	RR (ms)	QRS(ms)	BW (g)	HW (g)	Tibia lenght (TL) (cm)	HW/TL (g/cm)	Liver weight (g)	Lung weight (g)	Lung weight/TL (g/cm)
SHAM	120.5±22.3	9.05±2.4	27.9±3.7	0.16±0.03	1.8±0.06	0.089±0.02	1.2±0.2	0.18±0.02	0.099±0.01
TAC	111.6±18.4	11.47±2.7*	29.4±4.8	0.25±0.05*	1.77±0.3	0.15±0.05*	1.3±0.3	0.2±0.02*	0.123±0.04

Table 1: ECG and organ weights in SHAM/TAC AC3-I mice

- **A)** Comparison of ECG RR and QRS, as well as anatomic weights between SHAM and TAC AC3-I mice. *=P<0.05 compared to SHAM.

CaMKII inhibition by AC3-I positively influences conduction velocity
Under SHAM conditions, AC3-I mice had significantly higher conduction velocities (both transversal and longitudinal) than WT mice, both in the left and right ventricle. Moreover, where WT mice showed a significant decrease in longitudinal conduction velocity upon TAC, AC3-I TAC mice had a preserved conduction velocity, with no significant differences between SHAM and TAC conditions. (Fig 3 and table 2).

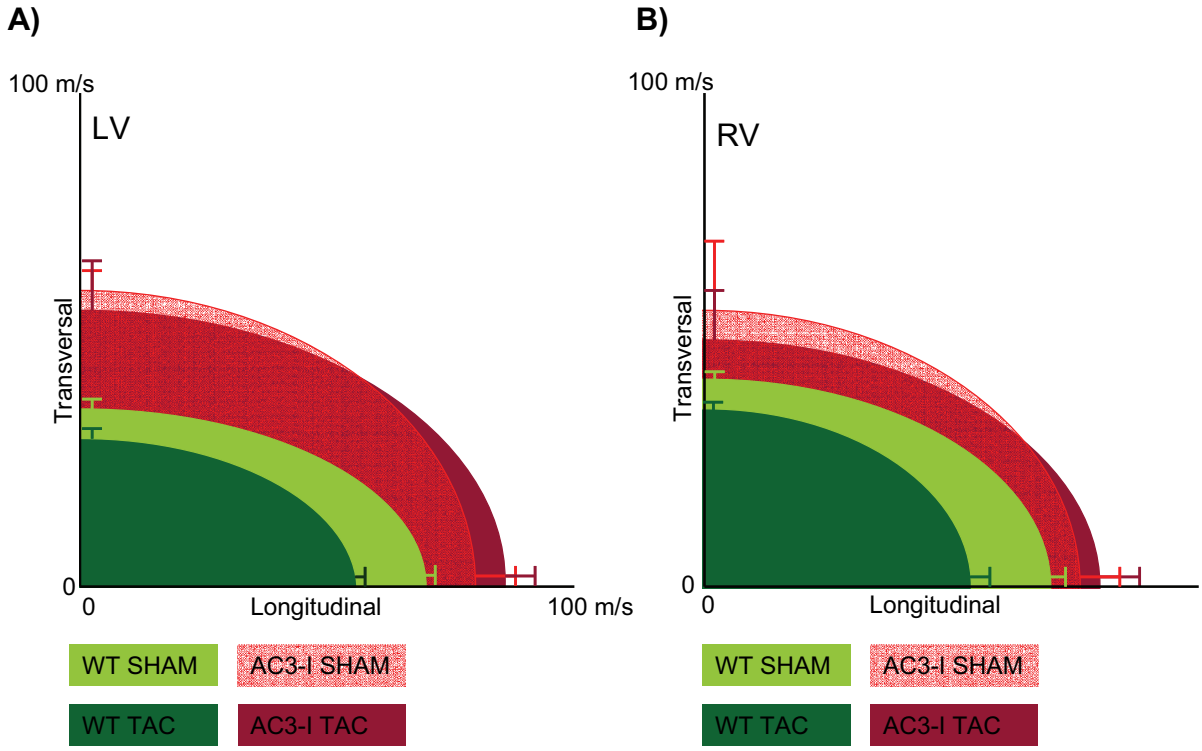


Figure 3: CaMKII inhibition positively influences conduction velocity
A) Mean±SD conduction velocity in the LV. On the X axis longitudinal, and on the Y axis transversal velocity. WT conduction velocity is depicted in green. After TAC (dark green) conduction slowed significantly. Conduction velocity in AC3-I mice is depicted in red/purple. TAC (purple) did not decrease conduction velocity, and SHAM as well as TAC velocities were higher compared to WT.
B) Idem, but for the right ventricle.

		LV (m/s)		RV (m/s)	
		CV Longitudinal	CV Transversal	CV Longitudinal	CV Transversal
WT	SHAM	70.4±5.5	35.5±1.8	71.5±6.9	42.2±2.1
	TAC	56±1.7*	30.6±1.7	54.2±8.0*	36.3±1.9
AC3-I	SHAM	79.7±11.5 #	60±6.7 #	75.6±10 #	55.1±9.7 #
	TAC	86.2±6.7 #	53.5±11 #	80.7±11 #	50.6±17.5 #

Table 2: Conduction velocities in WT and AC3-I mice
Mean±SD conduction velocities (meters/second), longitudinal and transversal, in the right and left ventricle, both in WT and AC3-I mice. *=P<0.05 compared to SHAM, #=P<0.05 compared to WT.

AC3-I TAC mice are still arrhythmic

Despite the preservation of conduction velocity, AC3-I mice still showed arrhythmias on the Langendorff set-up after TAC (SHAM mice showed no arrhythmias). Arrhythmias were pacing induced, polymorphic, and sustained (>15 beats), like the example given in figure 4A. The percentage of TAC mice with arrhythmias was comparable to WT proportions, like in the study of Jansen et al.[2] or Boulaksil et al.[14] (see: Fig 4B).
Importantly, the epicardial activation maps generated during the arrhythmias showed no signs of structural block (only 1 extra beat showed signs of re-entry during epicardial mapping, probably due to functional block), and conduction seemed to be preserved in general (Fig 4C).

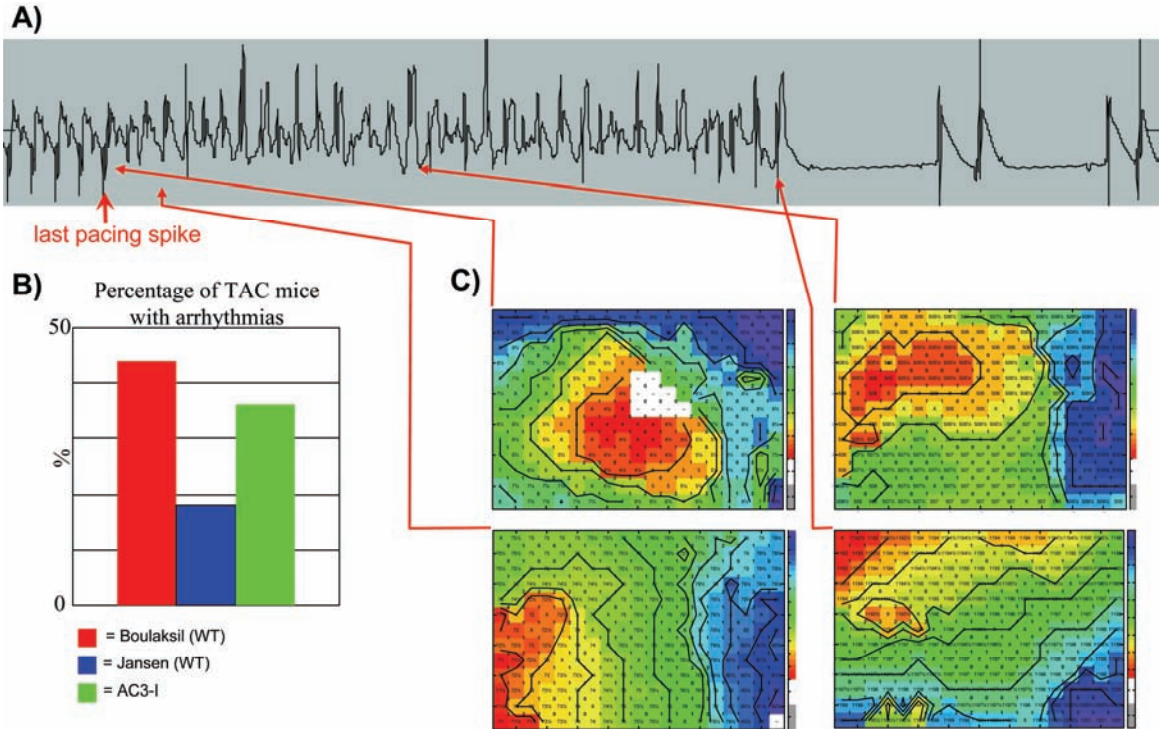


Figure 4: Arrhythmias in TAC AC3-I mice are polymorphic, pacing induced, and have unstable epicardial mappings

- **A)** Example of a pacing induced polymorphic ventricular tachycardia in a TAC operated AC3-I mouse
- **B)** Comparison of the percentage of TAC AC3-I mice with arrhythmias to two other studies with WT mice
- **C)** Examples of epicardial mappings of arrhythmic beats in TAC AC3-I mice. Arrows link the epicardial map to the measured beat.

AC3-I mice do not show a reduction in ventricular Cx43 and Nav1.5 expression, but fibrosis is increased after TAC

A number of molecular markers are known to influence conduction velocity, like functionality of Nav1.5 composed sodium channels and Cx43 built gap junction channels, as well as fibrosis. Cx43 expression pattern in AC3-I mice showed no changes between the SHAM and TAC group. Both in the left and right ventricle, Cx43 expression, as determined via immunohistochemistry, remained unchanged (Fig 5). Nav1.5, similarly, was also not significantly altered after TAC in AC3-I mice (Fig 6). Fibrosis, in contrast, did show profound changes when assessed through PicroSirius Red staining. AC3-I mice displayed areas of patchy fibrosis after TAC (Fig 7A), and total fibrosis levels were significantly increased (Fig 7B) when compared to SHAM.

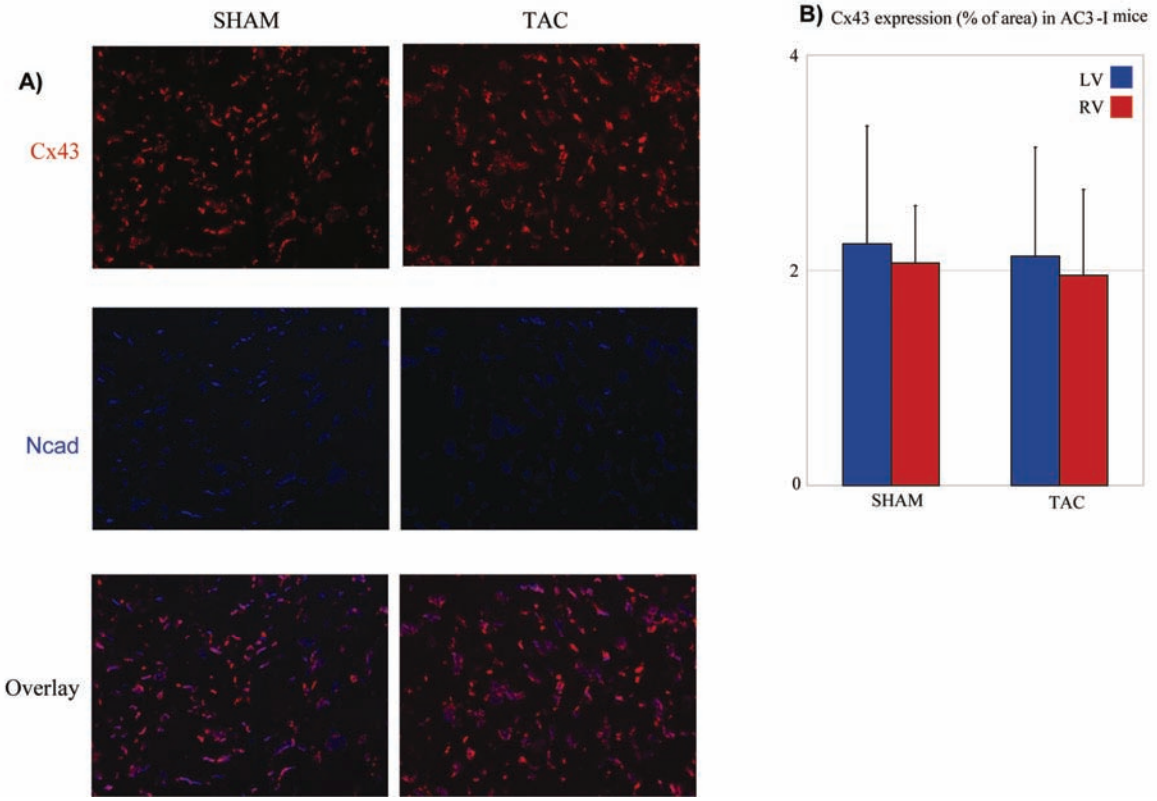


Figure 5: AC3-I mice have preserved Cx43 expression

A) Examples of immunohistochemistry against Cx43, and Ncad as a control for intercalated discs, in SHAM and TAC AC3-I mice

B) Quantification of immunohistochemistry as % of total area.

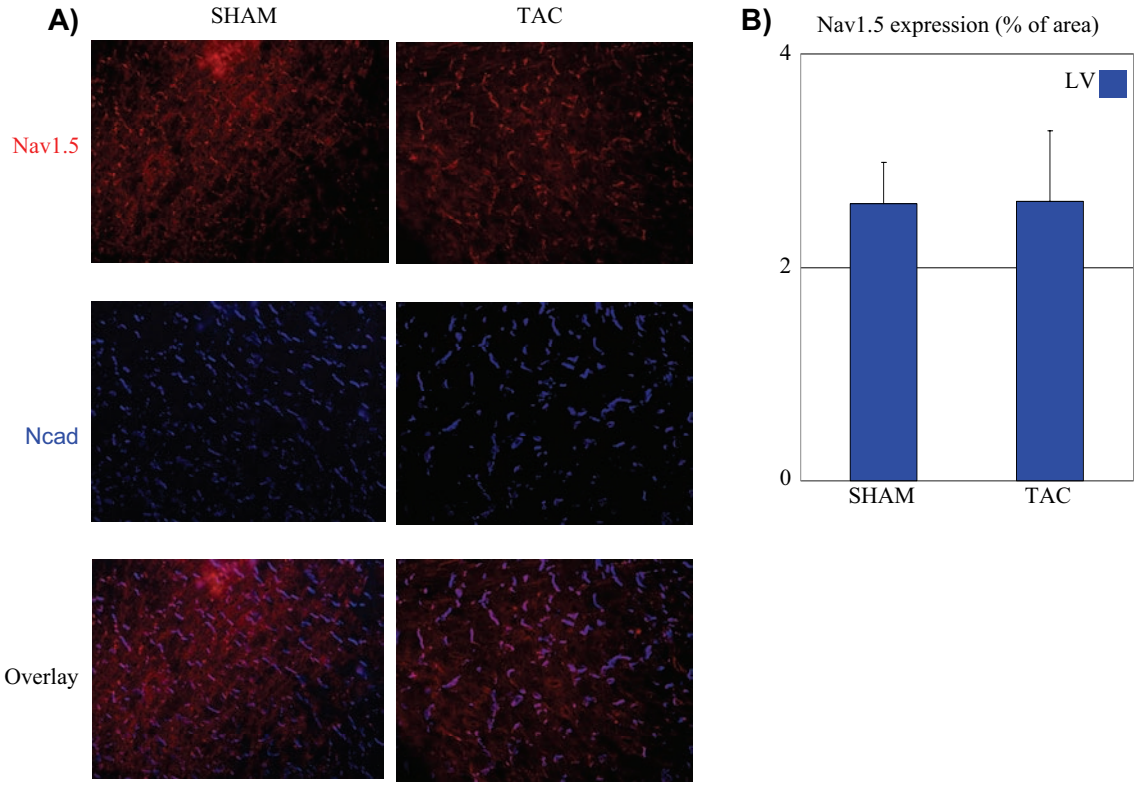


Figure 6: Nav1.5 expression in AC3-I mice

A) Examples of immunohistochemistry against Cx43, and Ncad as a control for intercalated discs, in SHAM and TAC AC3-I mice

B) Quantification of immunohistochemistry as % of total area.

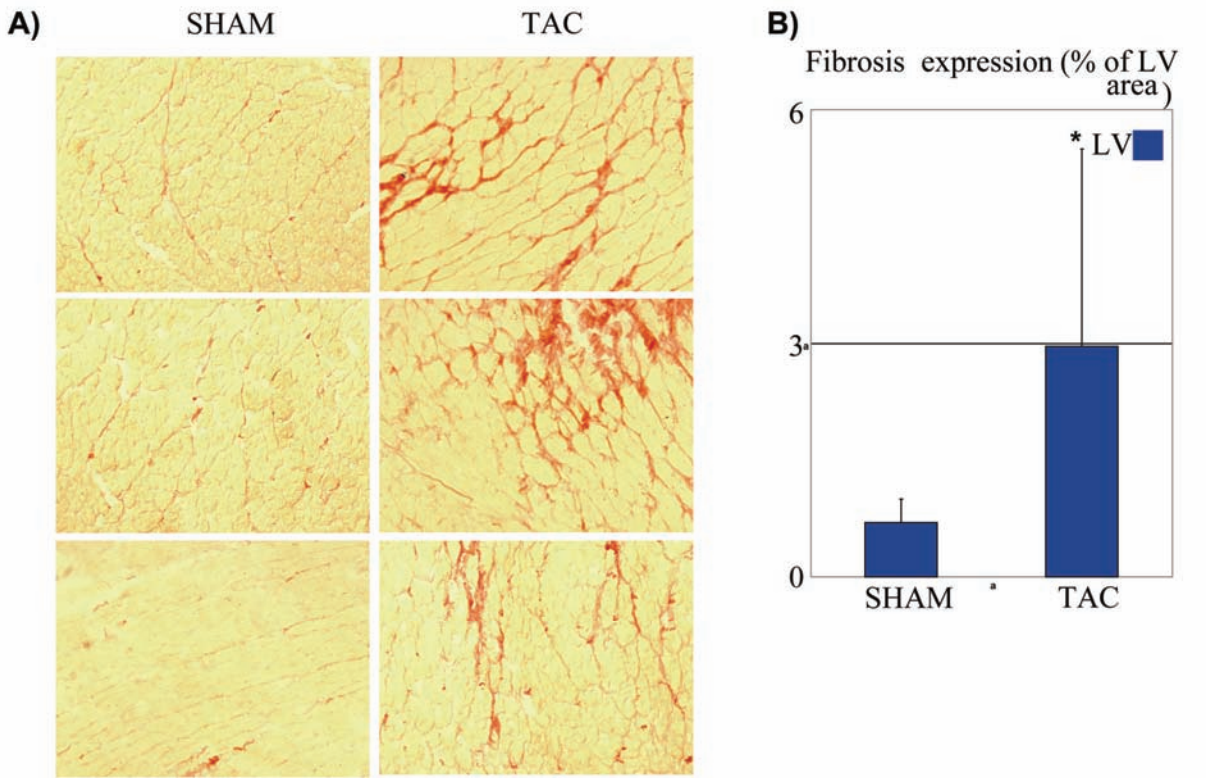


Figure 7: CaMKII inhibition does not diminish fibrosis accumulation after TAC
A) Examples of Sirius red stained tissue sections of SHAM or TAC treated AC3-I mice
B) Quantification of Sirius red staining.

Discussion

In this study we show that chronic CaMKII inhibition by AC3-I was able to prevent slowing of conduction velocity during pressure overload induced cardiac remodeling. However, arrhythmias were still present in a percentage comparable to that in WT-TAC mice. The preserved conduction velocity was accompanied by preserved Cx43 and Nav1.5 expression, but fibrosis levels were still increased after TAC. CaMKII inhibition in this TAC model did neither prevent development of hypertrophy nor the reduction in fractional shortening.

CaMKII and TAC induced hypertrophy

Numerous research groups all report the same effect of TAC in mice: hypertrophy, increased lumen diameters, and decreased fractional shortening[9, 13, 19-21, 23, 24], as well as CaMKII activation[9, 13, 20, 21, 24]. Our results are in agreement with this.
The complete lack of a protective effect of CaMKII inhibition against hypertrophy (heart weight corrected for body weight) and reduced cardiac function (fractional shortening) that we observed however, is in disagreement with previous published literature. Ling et al.[9], for example, observed a better fractional shortening in CaMKII knock out mice, while Lu et al.[20], described the same upon pharmaceutical CaMKII inhibition. There are a number of potential possibilities to explain this. First, AC3-I inhibition is markedly different than either pharmaceutical inhibition, which is less specific, and total knock-out, which could also disturb

protein complexes of which CaMKII is part. The largest difference between our study and others, however, is the time we have reserved to allow cardiac remodeling (16 weeks). All other studies named in this paragraph included periods ranging from a minimum of 7 to a maximum of 56 days. Perhaps it is more appropriate to say that CaMKII inhibition slows cardiac remodeling, but does not prevent it (at least as long as the trigger remains), especially since it is well known that other signaling proteins are also responsible for cardiac remodeling, like calcineurin[25].

CaMKII and conduction velocity

Very little is known about the effect of CaMKII on conduction velocity. Although numerous studies have been published that link CaMKII to arrhythmias, this is most often in a triggered activity setting. One study has simulated the effects of CaMKII activation on conduction velocity around the border zone in cardiac infarction, and suggests it slows conduction due to reduced sodium channel availability[26]. Unfortunately, experimental data were not provided. We are the first to show a link between CaMKII and cardiac conduction velocity. The question remains though, what the exact mechanistic link is between CaMKII and conduction. In previous work, our group has identified three factors underlying reduced conduction: decreased sodium current[18], less connexin-enabled cell coupling[27], and reduced cellular coupling because of increased fibrosis[28].

The effects of CaMKII inhibition cannot be explained by fibrosis, as this was not rescued by AC3-I. In contrast, Cx43 and Nav1.5 expression were not different between SHAM and TAC groups. A change in only one of these factors does not necessarily lead to a change in conduction velocity, but a combination of two may well have a significant effect[2, 18].

In neuronal tissue, CaMKII has been observed to decrease developmental connexin-dependent cellular coupling[29]. It has also been noted by our group and others that Cx43 and Nav1.5 expression in cardiomyocytes are closely intertwined[30]. A positive CaMKII effect on Cx43 expression and cellular coupling may thus translate in positive effects of Nav1.5 expression as well, and therefore provides a potential explanation for the preserved conduction velocity. One group has used a proteomics approach to identify CaMKII phosphorylation sites on Cx43, but the physiological relevance of this is not yet known[31]. Nav1.5 phosphorylation sites have also been identified, but have been linked to changes in gating properties, not expression levels[7].Whether CaMKII influences Cx43 and Nav1.5 expression directly or indirectly needs further clarification.

There are studies showing that the entire desmosomal protein complex is a carefully balanced structure, where changes in mechanical coupling also affects electrical coupling and Nav1.5 positioning in the intercalated disc[32]. Furthermore, CaMKII has indeed been connected to anchoring proteins. For example, one article[33] describes an interaction between CaMKII and SAP97, which is also involved in sodium channel lateralization[34]. However, also in this case, the exact mechanism requires further elucidation.

Conduction velocity and arrhythmias

The mechanisms behind ventricular arrhythmias can be roughly divided in two groups: triggered activity and re-entry based mechanisms. The first is dependent on afterdepolarizations, where cells are able to excite themselves[35]. The second mechanism occurs if severe conduction problems are present and there is a substrate for re-entry circuits to form[36].

In WT mice after 16 weeks of TAC-induced remodeling, conduction slowing is present, and indeed, it is possible to record re-entry circuits in epicardial mapped arrhythmias[14]. However, it is not clear whether re-entry is the sole cause of arrhythmias, or whether it is mainly important for perpetuation of triggered activity induced arrhythmias.

In the AC3-I TAC mice re-entry is unlikely. Firstly, conduction velocity is preserved, and secondly, in only one arrhythmic beat an epicardial mapping pattern resembling a re-entry circuit was observed, which was not present in the previous or following beats. All other arrhythmic beats showed signs of triggered arrhythmias, either by an activation front coming from a remote area and swiftly passing over the mapped epicardial surface, or originating from a focus right under the electrode grid.
Importantly, all arrhythmias induced in these mice followed after burst pacing, a technique which can induce calcium overload and therefore triggered activity. However, only 1 or 3 extra beats close to the effective refractory period, a method to induce re-entry circuits, was never effective. We therefore postulate that CaMKII inhibition in this setting might rescue re-entry based arrhythmogenic mechanisms, possibly involved in perpetuation of arrhythmias in TAC WT mice, but triggered activity based arrhythmias could not be prevented.

In conclusion, CaMKII inhibition increased conduction velocity and preserved Nav1.5, Cx43, but not fibrosis. This intervention appeared not anti-arrhythmic, possibly because arrhythmias were due to triggered activity rather than re-entry.

Reference List

(1) Boulaksil M, Winckels SK, Engelen MA et al. Heterogeneous Connexin43 distribution in heart failure is associated with dispersed conduction and enhanced susceptibility to ventricular arrhythmias. *Eur J Heart Fail* 2010 September;12(9):913-21.

(2) Jansen JA, Noorman M, Musa H et al. Reduced heterogeneous expression of Cx43 results in decreased Nav1.5 expression and reduced sodium current that accounts for arrhythmia vulnerability in conditional Cx43 knockout mice. *Heart Rhythm* 2012 April;9(4):600-7.

(3) Anderson ME, Braun AP, Wu Y et al. KN-93, an inhibitor of multifunctional Ca++/calmodulin-dependent protein kinase, decreases early afterdepolarizations in rabbit heart. *J Pharmacol Exp Ther* 1998 December;287(3):996-1006.

(4) Wu Y, Temple J, Zhang R et al. Calmodulin kinase II and arrhythmias in a mouse model of cardiac hypertrophy. *Circulation* 2002 September 3;106(10):1288-93.

(5) Khoo MS, Li J, Singh MV et al. Death, cardiac dysfunction, and arrhythmias are increased by calmodulin kinase II in calcineurin cardiomyopathy. *Circulation* 2006 September 26;114(13):1352-9.

(6) Anderson ME. Multiple downstream proarrhythmic targets for calmodulin kinase II: moving beyond an ion channel-centric focus. *Cardiovasc Res* 2007 March 1;73(4):657-66.

(7) Wagner S, Hacker E, Grandi E et al. Ca/calmodulin kinase II differentially modulates potassium currents. *Circ Arrhythm Electrophysiol* 2009 June;2(3):285-94.

(8) Zhang T, Johnson EN, Gu Y et al. The cardiac-specific nuclear delta(B) isoform of Ca2+/calmodulin-dependent protein kinase II induces hypertrophy and dilated cardiomyopathy associated with increased protein phosphatase 2A activity. *J Biol Chem* 2002 January 11;277(2):1261-7.

(9) Ling H, Zhang T, Pereira L et al. Requirement for Ca2+/calmodulin-dependent kinase II in the transition from pressure overload-induced cardiac hypertrophy to heart failure in mice. *J Clin Invest* 2009 May;119(5):1230-40.

(10) Boulaksil M, Winckels SK, Engelen MA et al. Heterogeneous Connexin43 distribution in heart failure is associated with dispersed conduction and enhanced susceptibility to ventricular arrhythmias. *Eur J Heart Fail* 2010 June 9.

(11) Boulaksil M, Noorman M, Engelen MA et al. Longitudinal arrhythmogenic remodelling in a mouse model of longstanding pressure overload. *Neth Heart J* 2010 October;18(10):509-15.

(12) Boulaksil M, Winckels SK, Engelen MA et al. Heterogeneous Connexin43 distribution in heart failure is associated with dispersed conduction and enhanced susceptibility to ventricular arrhythmias. *Eur J Heart Fail* 2010 June 9.

(13) Colomer JM, Mao L, Rockman HA, Means AR. Pressure overload selectively up-regulates Ca2+/calmodulin-dependent protein kinase II in vivo. *Mol Endocrinol* 2003 February;17(2):183-92.

(14) Boulaksil M, Winckels SK, Engelen MA et al. Heterogeneous Connexin43 distribution in heart failure is associated with dispersed conduction and enhanced susceptibility to ventricular arrhythmias. *Eur J Heart Fail* 2010 September;12(9):913-21.

(15) van Rijen HV, Eckardt D, Degen J et al. Slow conduction and enhanced anisotropy increase the propensity for ventricular tachyarrhythmias in adult mice with induced deletion of connexin43. *Circulation* 2004 March 2;109(8):1048-55.

(16) Jansen JA, van Veen TA, de Jong S et al. Reduced Cx43 expression triggers increased fibrosis due to enhanced fibroblast activity. *Circ Arrhythm Electrophysiol* 2012 April;5(2):380-90.

(17) Stein M, van Veen TA, Remme CA et al. Combined reduction of intercellular coupling and membrane excitability differentially affects transverse and longitudinal cardiac conduction. *Cardiovasc Res* 2009 July 1;83(1):52-60.

(18) van Veen TA, Stein M, Royer A et al. Impaired impulse propagation in Scn5a-knockout mice: combined contribution of excitability, connexin expression, and tissue architecture in relation to aging. *Circulation* 2005 September 27;112(13):1927-35.

(19) Backs J, Backs T, Neef S et al. The delta isoform of CaM kinase II is required for pathological cardiac hypertrophy and remodeling after pressure overload. *Proc Natl Acad Sci U S A* 2009 February 17;106(7):2342-7.

(20) Lu YM, Huang J, Shioda N et al. CaMKIIdeltaB mediates aberrant NCX1 expression and the imbalance of NCX1/SERCA in transverse aortic constriction-induced failing heart. *PLoS One* 2011;6(9):e24724.

(21) Toischer K, Rokita AG, Unsold B et al. Differential cardiac remodeling in preload versus afterload. *Circulation* 2010 September 7;122(10):993-1003.

(22) Potse M, Linnenbank AC, Grimbergen CA. Software design for analysis of multichannel intracardial and body surface electrocardiograms. *Comput Methods Programs Biomed* 2002 November;69(3):225-36.

(23) van Oort RJ, van RE, Bourajjaj M et al. MEF2 activates a genetic program promoting chamber dilation and contractile dysfunction in calcineurin-induced heart failure. *Circulation* 2006 July 25;114(4):298-308.

(24) Hsu S, Nagayama T, Koitabashi N et al. Phosphodiesterase 5 inhibition blocks pressure overload-induced cardiac hypertrophy independent of the calcineurin pathway. *Cardiovasc Res* 2009 February 1;81(2):301-9.

(25) Bierhuizen MF, Boulaksil M, van Stuijvenberg L et al. In calcineurin-induced cardiac hypertrophy expression of Nav1.5, Cx40 and Cx43 is reduced by different mechanisms. *J Mol Cell Cardiol* 2008 September;45(3):373-84.

(26) Christensen MD, Dun W, Boyden PA, Anderson ME, Mohler PJ, Hund TJ. Oxidized calmodulin kinase II regulates conduction following myocardial infarction: a computational analysis. *PLoS Comput Biol* 2009 December;5(12):e1000583.

(27) van Rijen HV, van Veen TA, van Kempen MJ et al. Impaired conduction in the bundle branches of mouse hearts lacking the gap junction protein connexin40. *Circulation* 2001 March 20;103(11):1591-8.

(28) Stein M, Boulaksil M, Jansen JA et al. Reduction of fibrosis-related arrhythmias by chronic renin-angiotensin-aldosterone system inhibitors in an aged mouse model. *Am J Physiol Heart Circ Physiol* 2010 August;299(2):H310-H321.

(29) Arumugam H, Liu X, Colombo PJ, Corriveau RA, Belousov AB. NMDA receptors regulate developmental gap junction uncoupling via CREB signaling. *Nat Neurosci* 2005 December;8(12):1720-6.

(30) Remme CA. Connexin43 and Na(V)1.5: partners in crime? *Heart Rhythm* 2012 April;9(4):608-9.

(31) Huang RY, Laing JG, Kanter EM et al. Identification of CaMKII phosphorylation sites in Connexin43 by high-resolution mass spectrometry. *J Proteome Res* 2011 March 4;10(3):1098-109.

(32) Noorman M, Hakim S, Kessler E et al. Remodeling of the cardiac sodium channel, Connexin43 and Plakoglobin at the intercalated disk in patients with arrhythmogenic cardiomyopathy. *Heart Rhythm* 2012 November 22.

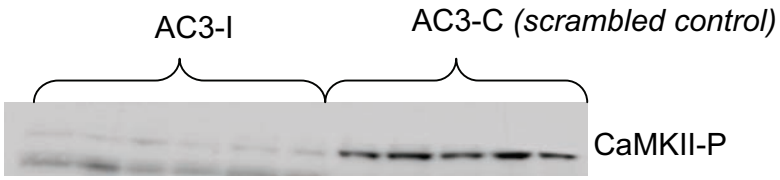
(33) El-Haou S, Balse E, Neyroud N et al. Kv4 potassium channels form a tripartite complex with the anchoring protein SAP97 and CaMKII in cardiac myocytes. *Circ Res* 2009 March 27;104(6):758-69.

(34) Petitprez S, Zmoos AF, Ogrodnik J et al. SAP97 and dystrophin macromolecular complexes determine two pools of cardiac sodium channels Nav1.5 in cardiomyocytes. *Circ Res* 2011 February 4;108(3):294-304.

(35) Pogwizd SM, Bers DM. Cellular basis of triggered arrhythmias in heart failure. *Trends Cardiovasc Med* 2004 February;14(2):61-6.

(36) Antzelevitch C. Basic mechanisms of reentrant arrhythmias. *Curr Opin Cardiol* 2001 January;16(1):1-7.

Supplemental figure: CaMKII autophosphorylation in mouse heart tissue



Chapter 6:

Chronic CaMKII inhibition is unable to prevent calcineurin-induced conductional remodeling of the heart.

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In preparation

Abstract

Background:
Calcineurin is a phosphatase involved in intracellular signaling leading to hypertrophy and heart failure when overactive. This phenotype is accompanied by arrhythmias and conduction slowing. The latter is based on increased fibrosis, less sodium channels, and decreased connexin-based intercellular coupling. A potential mediator of this molecular mechanism of conduction slowing is calcium/calmodulin-dependent protein kinase II (CaMKII), a signaling kinase that, once chronically active, can lead to detrimental cardiac hypertrophy.

Methods & Results
Interbreeding of mice overexpressing a continuously active form of calcineurin A (CnA) with transgenic mice overexpressing the specific CaMKII inhibitor AC3-I in the heart, resulting in the CnA*AC3-I strain, normalized cardiac CaMKII activity and decreased arrhythmia susceptibility, according to Khoo et al.[1] Since the molecular basis for the reduced arrhythmia susceptibility of CnA*AC3-I hearts is not known, we determined Nav1.5 and Cx43 expression at both the protein and the RNA level by immunoblotting and quantitative PCR, respectively. Moreover, the level of fibrosis was determined by picrosirius red staining, and in a pilot study ECGs and Langendorff pacing tests were added. In CnA*AC3-I hearts, Nav1.5 protein and RNA levels were significantly reduced, whereas for Cx43 the protein, but not RNA, level was decreased. The amount of fibrosis proved to be increased relative to wild type. Furthermore, elevated heart weights and increased QRS duration were not rescued. Pacing threshold was very high, suggesting conduction abnormalities.

Conclusion:
Chronic CaMKII inhibition is unable to prevent calcineurin-induced conductional remodeling of the heart.

Abbreviations:
AC3-C/I: autocamtide 3 related inhibitory (I) peptide (C= control, scrambled peptide)
CaMKII: calcium/calmodulin-dependent protein kinase II
CnA: calcineurin A
Cx: Connexin
GATA4: Gata binding protein 4
GFP: green fluorescence protein
MEF2: myocyte enhancer factor 2
MHC: myosin heavy chain
miR: microRNA
Nav1.5: voltage-gated sodium channel, type V, alpha subunit (protein)
NFAT: nuclear factor of activated T-cells
NP: non-phosphorylated
Scn5a: voltage-gated sodium channel, type V, alpha subunit (gene)
WT: wildtype

Introduction

In the western world cardiovascular disease is still the number one cause of mortality, whereas heart failure is the fastest growing subclass. The heart is able to remodel in response to changes in physiological demand (exercise or pregnancy) and several forms of cardiac pathology.[2, 3]
Cardiac diseases often lead to hypertrophy, for example due to sustained hypertension or myocardial infarction. Hypertrophy is an increase in the volume of individual cardiomyocytes, which in turn leads to increased pump function and a decrease of the stress on the ventricular wall.[2, 4] Longterm cardiac hypertrophy is related to an increased propensity for ventricular arrhythmias and eventually might result in sudden death.[5-7]
There are several intracellular signaling pathways involved in cardiac hypertrophy. One of the cascades involved, is the NFAT-mediated pathway.[4, 8] In this cascade the serine-threonine protein phosphatase CnA plays a major role. An increase of intracellular Ca^{2+} will cause binding to calmodulin, a Ca^{2+} binding protein. This Ca^{2+}-calmodulin complex is able to activate calcineurin which is a heterodimer. It consists of a catalytic CnA subunit and a regulatory calcineurin B subunit. The Ca^{2+}-calmodulin complex binds to the calmodulin binding domain in CnA, causing activation. When CnA is activated, members of the NFAT family are attracted and will be dephosphorylated, leading to translocation of NFAT from the cytosol to the nucleus. In the nucleus NFAT activates a hypertrophy gene program which also involves the transcription factors GATA4 and MEF2.[8-11]
To study the mechanism behind hypertrophy and arrhythmias in more detail Molkentin *et al.*[11] developed a transgenic mouse model, with a cardiac specific αMHC-promotor driven overexpression of a constitutively active form of CnA. These mice develop cardiac hypertrophy within 18 days in all four chambers that rapidly deteriorates in heart failure, extensive deposition of fibrosis and sudden death[2] between 3 and 12 weeks of age. Also they have a higher vulnerability for arrhythmias and an increased CAMKII activity[1].
Bierhuizen et al.[9] showed that mice overexpressing CnA have a prolongation of atrial and ventricular activation time with associated impaired excitability and conduction properties. The reduction in conduction velocity was correlated with reductions in expression of the sodium channel Nav1.5 and the gap junction protein Cx43. Alterations that are also reported in human heart failure.[11, 12]
In addition to the CnA overexpressing mouse model, the group of Anderson developed a transgenic mouse model in which CaMKII, a serine/threonine kinase that phosphorylates multiple target proteins, is cardiac specifically inhibited through overexpression of a specific CaMKII-inhibitory peptide (AC3-I).[1] When these mice were crossed with the CnA overexpressing mice, the downregulation of CaMKII activity in the heart was accompanied by decreased mortality, suppression of arrhythmias and improvement in left ventricular function as compared to the CnA*AC3-C mice, the corresponding control. Considering the association of decreased Nav1.5 and Cx43 expression with increased arrhythmia susceptibility,[13, 14] the suppression of arrhythmias upon CaMKII inhibition in the CnA model suggested a role for CaMKII in conductional remodeling.
To assess the potential effects on the three main factors involved in conduction[14-16], levels of connective tissue, and of Cx43 and Nav1.5 (both protein and RNA) were determined in ventricles of WT, CnA, CnA*AC3-C and CnA*AC3-I mice. In this study we provide evidence that CaMKII inhibition did not normalize levels of Nav1.5, Cx43, and connective tissue in calcineurin-induced hypertrophy.

Methods

Animals
Mouse hearts (N=4 per group) were kindly provided by Dr. M.E. Anderson (University of Iowa, U.S.A). The following genotypic groups were used:
- WT
- α myosin heavy chain driven overexpression of a constitutively active form of CnA (CnA)
- Overexpression of a CaMKII inihibitory peptide (AC3I)
- Overexpression of a scrambled non-functional CaMKII inhibitory peptide (AC3C)
- Combined overexpression of CnA and AC3-I (CnA*AC3-I)
- Combined overexpression of CnA and AC3-C (CnA*AC3-C)

Both the CamKII inhibitory peptide as well as the control was tagged with green fluorescent protein. Additionally, AC3-I mice from the Anderson-lab were used to set up a cross breeding with CnA mice from our group, received from Dr. E.N. Olson.[11] Specifically, offspring was used from CnA males breeded with AC3-I females. Breeding was performed in a C57/BL6 background. All animal experiments were approved by the institutional ethical committee for animal experiments of the UMC Utrecht.
Genotypic characterization was performed as described in the supplemental materials (supplemental figure 1 and 2).

Western blotting
Ventricular samples were pulverized using a precooled steel mortar and subsequently lysed with RIPA lysis buffer. Isolated protein (20μg/lane) was used for SDS-PAGE and subsequent Western blotting. Chemiluminescence was performed using the ECL kit from Amersham. The following antibodies were used: anti-N-cadherin (Sigma), anti-β-catenin (Transduction), anti-Cx43 (Invitrogen-Zymed), anti-non phosphorylated Cx43 (Invitrogen-Zymed), anti-Nav1.5 (Alomone Labs), anti-Cn panA (Millipore) and anti-GFP (Santa Cruz).

Immunohistochemistry
10 μm-thick four chamber view tissue sections from different levels of the heart were labeled with anti-Cx43 (Invitrogen-Zymed). Levels of fibrosis were determined via picrosirius red staining. ImageJ software was used to quantify both Cx43 and fibrosis as percentage of total tissue area.

Quantitative PCR
RNA from ventricular samples was isolated using the Trizol procedure (Invitrogen). After reverse transcription expression levels were determined by quantitative PCR (Bio-Rad iCycler machine), using iQ SYBR Green Supermix (Bio-Rad). Details of the primers used can be found in supplemental table 1.

ECG, Langendorff perfusion, and epicardial mapping
CnA*AC3-I mice were used for ECG analysis and Langendorff perfusion. Experiments were performed blind, as characterization of the hearts was performed afterwards, via CnA and GFP (tag for AC3-I) western blots (see supplemental figure 3).
Mice were anesthetized (induction with 5% isoflurane, thereafter 2%) and three lead ECGs were recorded. After this, hearts were excised and connected to a Langendorff perfusion set-up which allowed mapping of epicardial activation as described in Bierhuizen et al.[9]
In short, hearts were continuously perfused with carbogen gassed buffer and an electrode grid was placed on the epicardial surface (19x13 electrodes, at 0.3 mm intervals). The procedure consisted of three stages: 1) sinus rhythm measurement, 2) programmed electrical stimulation

from a central electrode at twice the stimulation threshold voltage where a basic cycle length of 100-120ms was followed by a premature stimulus. Maximum stimulation voltage was 50 volt. 3) Burst pacing was performed at the shortest possible interval.
Analysis of ECGs was performed offline with Labchart7 software (AD instruments). Epicardial electrograms were also analysed offline with Matlab7.1 software (MathWorks), where the maximal –dV/dt was considered to be the moment of tissue activation directly underlying the electrode. To prevent interference of remote signals, the Laplacian was checked to confirm the –dV/dt was a local signal.

Statistical analysis
One-way analysis of variance with Bonferroni post hoc analysis was used. All data are expressed as the mean ± standard error of the mean: $P < 0.05$ was considered statistically significant.

Results

In vitro:

RNA expression for Scn5a and Cx43 in CnA, CnA*AC3-C and CnA*AC3-I hearts
First, we investigated the RNA levels using qPCR with specific primers (see supplemental table 1 for the sequences) against Scn5A, Cx43, CnA, and normalized against the internal control GAPDH. Figure 1 depicts the results obtained with samples of WT, CnA, CnA*AC3-C and CnA*AC3-I mice. For Scn5a a significant decrease was detected to, respectively 28%, 13%, and 23% the level of WT. A minor decrease was also seen for Cx43 to respectively 83%, 60%, and 71% compared to the level of WT but only the differences in AC3-C reached statistical significance. As expected, CnA expression was strongly increased in CnA, CnA*AC3-C, and CnA*AC3-I hearts. Interestingly, the expression of Scn5a and Cx43 was not different between CnA*AC3-C and CnA*AC3-I (Figure 1).

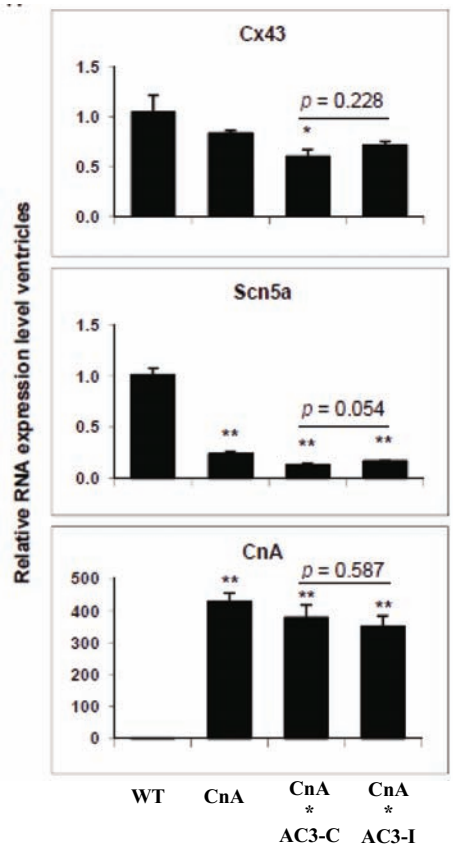

Figure 1: Q-PCR analysis on ventricular samples for Cx43, Scn5a, and CnA. The graphs show relative quantifications. *=P<0.05, **=P<0.01 compared to WT

Downregulation of Nav1.5 and Cx43 protein expression in CnA, CnA*AC3-C and CnA*AC3-I hearts

To investigate whether CaMKII is involved in the downregulation of Nav1.5 and Cx43 immunoblotting was performed on lysates from WT, CnA, CnA*AC3-C, and CnA*AC3-I (Figure 2). A statistically significant downregulation to 37%, 21%, and 13% of the WT level was seen in the expression of Nav1.5 in CnA, CnA*AC3-C and CnA*AC3-I, respectively. A similar reduction was observed for Cx43 to 58%, 45%, and 54% in hearts of CnA, CnA*AC3-C and CnA*AC3-I mice, respectively, relative to WT. No significant changes were observed in the expression of the junctional proteins β-catenin and N-cadherin. In contrast, a relative increase of Cx43-NP of respectively 3.71, 2.47, and 3.37 fold was seen. For CnA and CnA*AC3-I this increase was statistically significant. There was no significant difference in the expression of Nav1.5 (p=0.135), Cx43 (p=0.375) and Cx43-NP (p=0.152) between CnA*AC3-C and CnA*AC3-I. The results of the Cx43 immunoblot indicated that there was a shift from the highly phosphorylated state of Cx43 (upper or P2 band) to the non-phosphorylated state of Cx43 (lower or P0 band). As mentioned, this effect was also confirmed with the specific antibody (Cx43-NP) against the P0 isoform of Cx43.

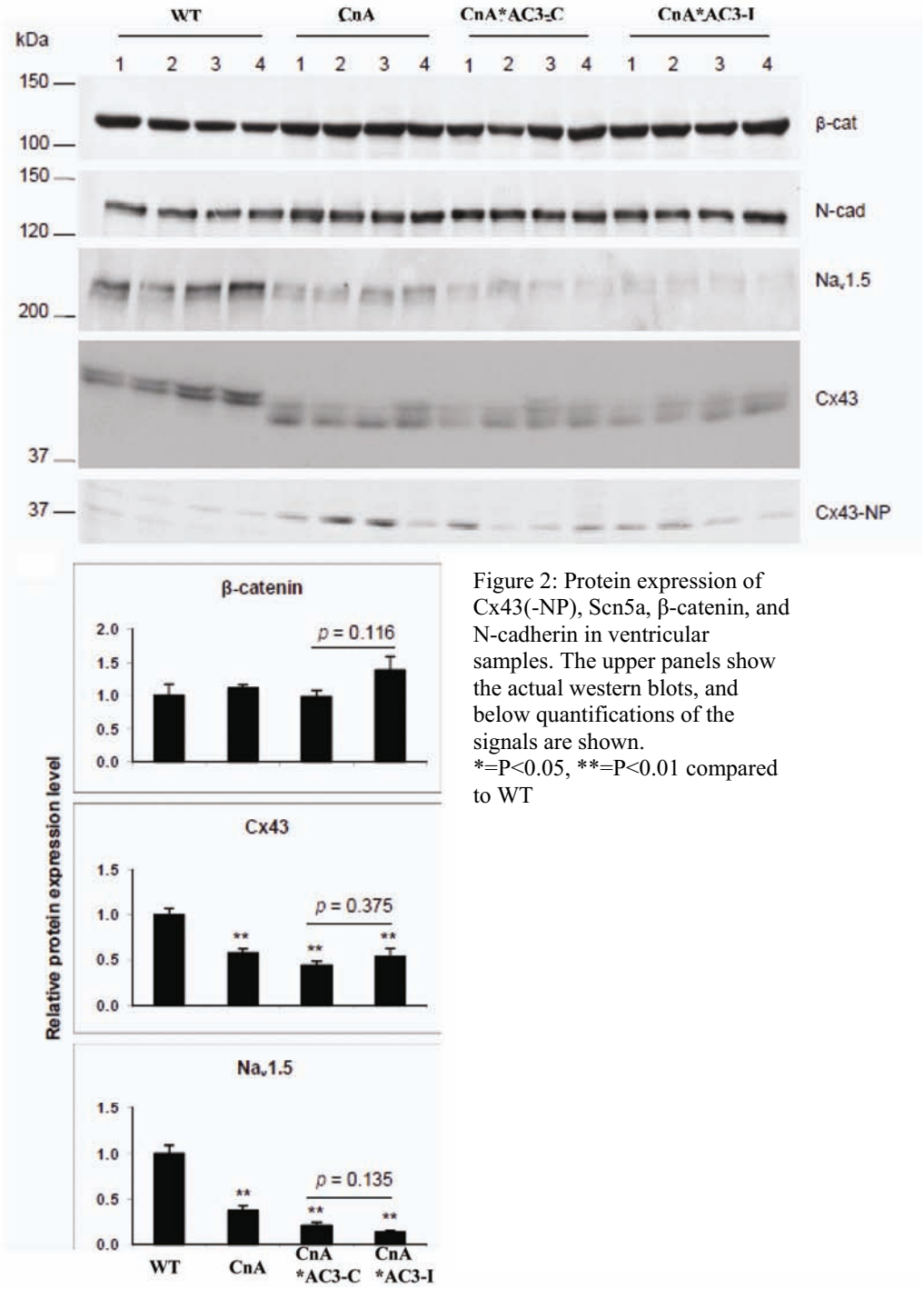

Figure 2: Protein expression of Cx43(-NP), Scn5a, β-catenin, and N-cadherin in ventricular samples. The upper panels show the actual western blots, and below quantifications of the signals are shown. *=P<0.05, **=P<0.01 compared to WT

Immunohistochemical analysis of Cx43 protein in tissue sections

To investigate if the reduction of Cx43 could be confimed in tissue sections, immunohistochemistry was performed. In Figure 3A representative examples of Cx43 expression in the different groups are illustrated and the overall quantification is shown to the right of it. The results indicate that there was a statistically significant reduction in all 3 groups to approximately 40% compared to WT, both in the left as well as right ventricle. No significant difference could be detected between CnA*AC3-I and CnA*AC3-C.

Connective tissue remodeling in AC3-I hearts

Previously, it was shown that the CnA mice showed an extensive increase in cardiac interstitial fibrosis.[11] To determine the presence of fibrosis in the mice hearts we studied, tissue sections were stained with picrosirius red. Figure 3B illustrates representative examples of such staining: collagen stains red and tissue yellow. An increase of interstitial fibrosis was observed which was homogenously present in the left and right ventricles. To the right of the pictures, quantification of the picrosirius red staining is shown: a statistically significant increase was seen of interstitial collagen in the CnA, CnA*AC3-C, and CnA*AC3-I mice relative to WT; the extent of this staining was not different between CnA*AC3-C and CnA*AC3-I.

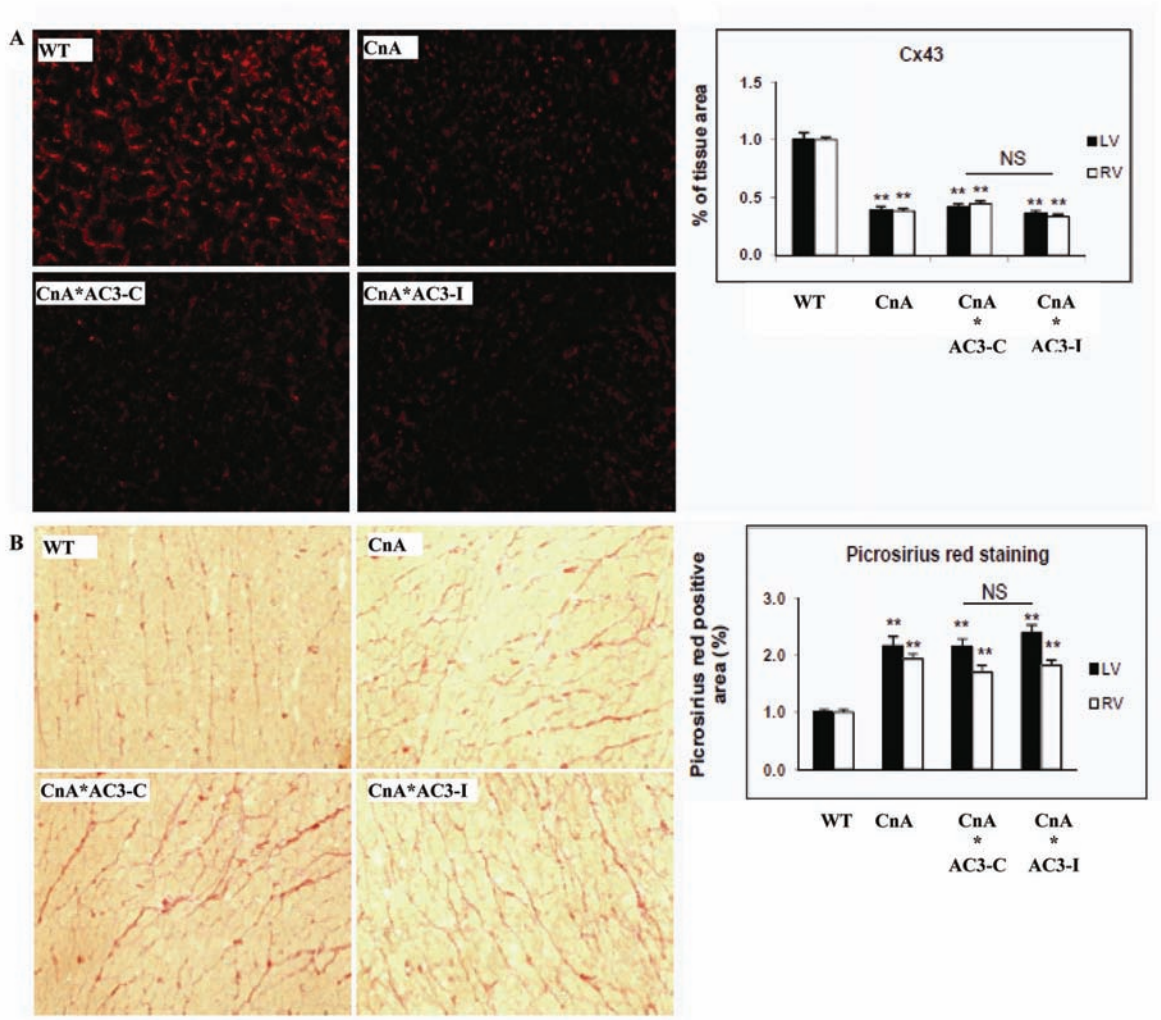

Figure 3: Histological analysis of Cx43 and fibrosis. A) immunohistochemistry of Cx43, with a quantification to the right. B) fibrosis as assessed via picrosirius red staing.
**=P<0.01 compared to WT. NS= non-significant, LV= left ventricle, RV= right ventricle.

In vivo ECG and *ex vivo* Langendorff perfusion experiments confirm lack of a CaMKII-dependent rescue of conduction parameters

To relate the above expressed *in vitro* results to conduction velocity we performed, in a small pilot experiment, ECG analysis and Langendorff perfusion with epicardial mapping. AC3-I (without CnA) and WT mice had comparable heart weight to tibia length ratios, indicating CaMKII inhibition alone had no significant effect on hypertrophy. Calcineurin is known to induce hypertrophy[17], as we also observed here both in presence or absence of CaMKII inhibition (Figure 4A).
In order to determine potential conduction abnormalities, ECG analysis was performed and QRS duration was determined. We also tried to perform epicardial mapping with a 19*13 electrode grid, where a central electrode functioned as pacing site. The delay in activation over the epicardium is a measure for conduction. Increased QRS width indicated slowed conduction in mouse 1 (CnA*AC3-I) and 7 (CnA) (Figure 4B). Epicardial pacing in both of these mice was impossible as the stimulation threshold was above 50 volt, which can be considered as an indicator for severe conduction problems. In the other mice (all WT or AC3-I without CnA) stimulation threshold was below 50 volt, but no sustained ventricular tachycardias could be induced (see figure 4C for an example).

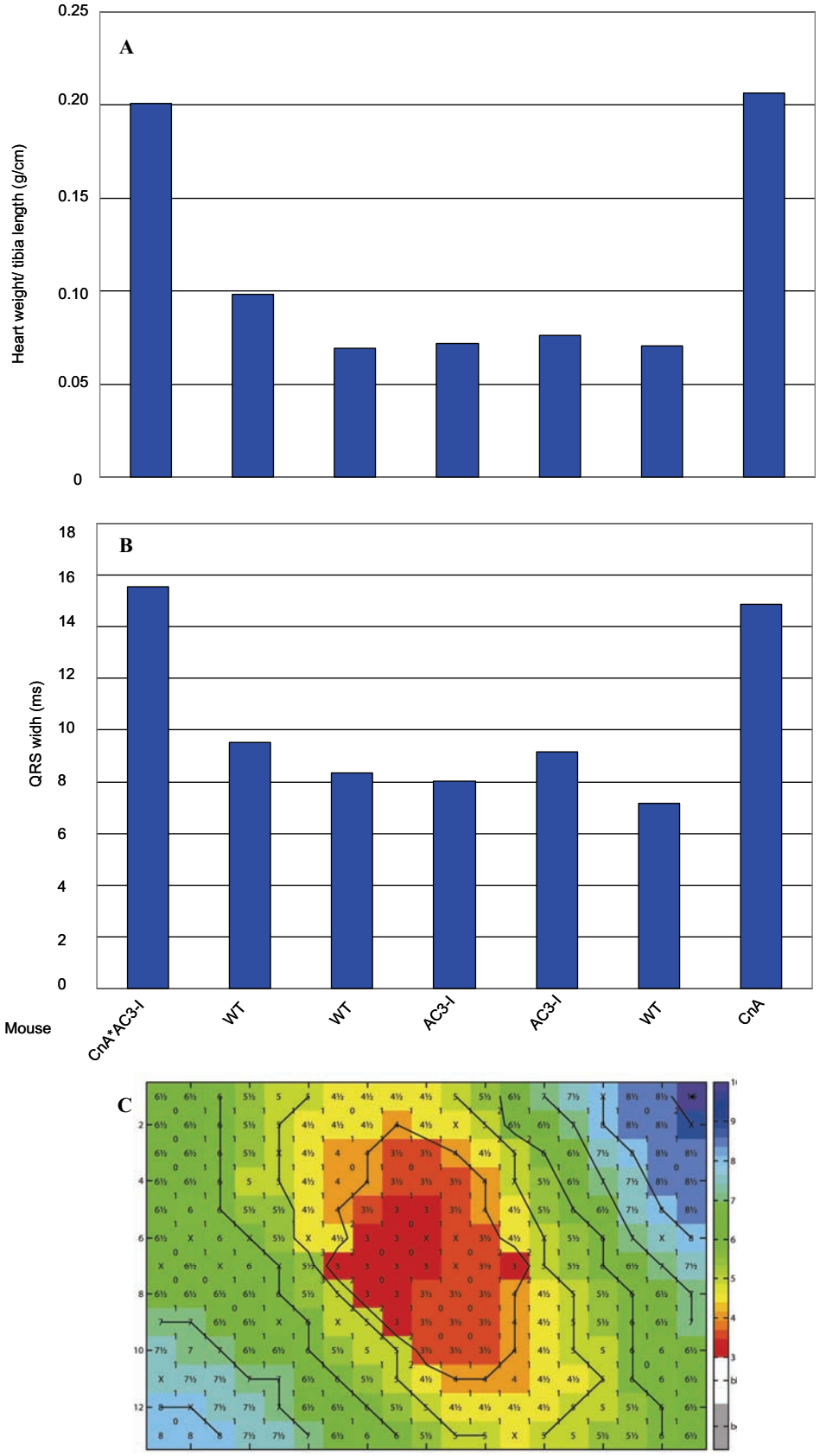

Figure 4: A) Heart weight to tibia length in the mice characterized in supplemental figure 3.
B) QRS width on the ECG in the same mice. C) Example of epicardial mapping in a WT mouse.

Discussion

Proper ventricular conduction is dependent on three factors: 1. tissue excitability, which is determined by Nav1.5 functionality; 2. cell-cell coupling, dependent on the gap junction protein Cx43 which forms electrical connections between the cardiomyocytes; and 3. the amount of connective tissue. Changes in these factors can eventually lead to an increased vulnerability to arrhythmias. In previous research Bierhuizen et al.[9] showed that the cardiac sodium channel Nav1.5 and the gap junction protein Cx43 were downregulated in CnA transgenic mice. Experiments in other models have also shown that a reduction of Nav1.5, Cx43, and an increase of fibrosis are involved in impaired electrical conduction which may lead to ventricular arrhythmias.[9, 13, 14, 16] However, until now the mechanisms underlying the remodeling of these conductional parameters have not been resolved.

From work of the Anderson lab[1] it appeared that CaMKII inhibition resulted in reduced arrhythmogenesis in CnA-induced hypertrophy and therefore it can be postulated that this intervention normalized the observed remodeling in Nav1.5, Cx43, and connective tissue. To study this, CnA*AC3-I and CnA*AC3-C hearts of the Anderson lab were used. The CnA*AC3-I and CnA*AC3-C hearts showed, similarly as for CnA, a reduction of Nav1.5 and Cx43 protein expression. Previously, at the RNA level Bierhuizen et al.[9] showed a reduction of Scn5A, but not Cx43, in the CnA transgenic hearts. Investigating the effect of CaMKII inhibition on RNA levels in the CnA*AC3-I mice, similar results as for CnA hearts were found. These results indicate that CaMKII inhibition has no effect on the downregulation of Nav1.5 and Cx43 expression in CnA hearts. Using Picrosirius red staining to study collagen deposition it appeared that CaMKII is also not involved in the development of fibrosis as there was a comparable amount of fibrosis in the CnA*AC3-I hearts compared to CnA and CnA*AC3-C. So, from these data we have to conclude that CaMKII inhibition did not normalize the calcineurin-mediated Nav1.5, Cx43, and connective tissue remodeling. Still, previous data suggest that in this model, CaMKII inhibition results in reduced arrhythmogeneity[1]. Our results suggest that, in this model, the anti-arrhythmic effect of CaMKII inhibition is not caused by normalization of the molecular factors underlying electrical conduction. This leads to the following question: what is reducing the arrhythmias and mortality in these CnA*AC3-I mice, if it is not via these molecular parameters? The arrhythmias may be either triggered or re-entry based. Triggered arrhythmias are linked to altered Ca^{2+} homeostasis and re-entry is dependent on alterations of conduction velocity and refractory period.[18] Possibly, AC3-I might lead to a reduction in triggered activity, as CaMKII is known to phosphorylate phospholamban[19], the L-type calcium channel[20], and the ryanodine receptor[21], all reported to be involved in delayed afterdepolarizations[22]. To assess this, cellular studies will have to be performed, where cells from CnA*AC3-I mice need to be patch clamped to determine membrane potential fluctuations. However, the non-rescue of Cx43, Nav1.5, and fibrosis, as well as the difficulty of pacing these hearts epicardially, and their broadened QRS complexes, suggests that re-entry is the more likely mechanism of arrhythmias.

More controversial, this conclusion might also lead to the explanation that CaMKII inhibition is actually not, or at best mildly, anti-arrhythmic in this model, which seems to be in contrast to Khoo et al.[1] However, the anti-arrhythmic effect observed by Khoo et al. is mild, with only a slight reduction in pacing-induced, but not spontaneous, arrhythmias. In such a line of thought, the lack of effect of CaMKII inhibition on Cx43, Nav1.5, and fibrosis, fits with a lack of anti-arrhythmic potential.

This suggests that the CnA induced cardiac remodeling in this model is independent of CaMKII. This is surprising, as there are studies suggesting a link between calcineurin and CaMKII[1, 23, 24], and increased CaMKII is to be expected in a DCM phenotype[25]. That

CaMKII, once chronically active, can induce cardiac remodeling culminating in DCM, is also reasonably well established[21, 26].
Potential explanations include a mechanism where CnA leads to DCM without requiring CaMKII. In this scenario CnA and CaMKII both lead to DCM, but in independent signaling cascades. Otherwise, it could be that with other triggers, like transverse aortic constriction or myocarditis, CaMKII inhibition could have a protective effect, but that in this case the overexpression of CnA is such an extreme trigger that this effect is lost.

The CnA-overexpressing mouse appears a good model to study the molecular mechanism behind the downregulation of Nav1.5 and Cx43 expression. It was suggested that the reduction of Cx43 occurs via a post-transcriptional mechanism and Nav1.5 via a transcriptional mechanism.[9] There are several possible post-transcriptional mechanisms which can be involved in Cx43 downregulation. First, direct dephosphorylation of Cx43 by CnA may lead to the degradation of Cx43; this can be a possible mechanism because our results show that there is indeed an increase of non-phosphorylated Cx43 in CnA, CnA*AC3-C, and CnA*AC3-I hearts. Cruciani et al.[27] investigated dephosphorylation of Cx43 by several phosphatases and suggested a poor interaction for CnA and Cx43 in hamster fibroblasts. In a test-tube experiment we showed that purified CnA was able to dephosphorylate Cx43, immunoprecipitated from mouse heart (results not shown).
Secondly, in literature it is described that impairment in the expression of desmosomal and adherens junction proteins like N-cadherin[28], desmoplakin[29], plakoglobin[30], plakophillin-2[31] and zonula occludens-1[32] are associated with the downregulation of Cx43 protein expression. Our group studied these desmosomal and adherens junction proteins in the CnA model and no alterations were found in expression that could explain the down-regulation of Cx43 in CnA relative to WT. Thirdly, the regulation of Cx43 by microRNAs may result in its reduced expression. Anderson et al.[33] showed that increased miR-1 and miR-206 expression in the skeletal myoblast resulted in a downregulation of Cx43 protein expression without affecting Cx43 mRNA levels. Van Rooij et al.[34] showed a set of miRs which were up or downregulated in the CnA model, but miR-206 and miR-1 changes were not reported. In contrast, in a recent study miR expression levels were also investigated in the CnA mouse model and in this study indeed miR-206, but not miR-1, was found to be upregulated.[35]
The transcriptional pathway which is causing the downregulation of Nav1.5 has not been revealed, but involvement of NFAT appears likely. This could, for example, be assessed via pharmacological NFAT inhibition, or via a NFAT knock-out mouse model[6]. In all, a number of potential pathways remain to be investigated in order to reveal the link of CnA with Cx43 and Nav1.5.

In summary our results indicate that, in the CnA*AC3-I mice, CnA induces deterioration of conduction velocity parameters, and CaMKII inhibition does not rescue the molecular factors underlying electrical conduction in this model.

Acknowledgements

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Reference List

(1) Khoo MS, Li J, Singh MV et al. Death, cardiac dysfunction, and arrhythmias are increased by calmodulin kinase II in calcineurin cardiomyopathy. *Circulation* 2006 September 26;114(13):1352-9.

(2) Hunter JJ, Chien KR. Signaling pathways for cardiac hypertrophy and failure. *N Engl J Med* 1999 October 21;341(17):1276-83.

(3) Schlossarek S, Carrier L. The ubiquitin-proteasome system in cardiomyopathies. *Curr Opin Cardiol* 2011 May;26(3):190-5.

(4) Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol* 2006 August;7(8):589-600.

(5) Adabag AS, Luepker RV, Roger VL, Gersh BJ. Sudden cardiac death: epidemiology and risk factors. *Nat Rev Cardiol* 2010 April;7(4):216-25.

(6) Bourajjaj M, Armand AS, da Costa Martins PA et al. NFATc2 is a necessary mediator of calcineurin-dependent cardiac hypertrophy and heart failure. *J Biol Chem* 2008 August 8;283(32):22295-303.

(7) Levy D, Anderson KM, Savage DD, Balkus SA, Kannel WB, Castelli WP. Risk of ventricular arrhythmias in left ventricular hypertrophy: the Framingham Heart Study. *Am J Cardiol* 1987 September 1;60(7):560-5.

(8) Heineke J, Auger-Messier M, Correll RN et al. CIB1 is a regulator of pathological cardiac hypertrophy. *Nat Med* 2010 August;16(8):872-9.

(9) Bierhuizen MF, Boulaksil M, van Stuijvenberg L et al. In calcineurin-induced cardiac hypertrophy expression of Nav1.5, Cx40 and Cx43 is reduced by different mechanisms. *J Mol Cell Cardiol* 2008 September;45(3):373-84.

(10) Bueno OF, van RE, Molkentin JD, Doevendans PA, De Windt LJ. Calcineurin and hypertrophic heart disease: novel insights and remaining questions. *Cardiovasc Res* 2002 March;53(4):806-21.

(11) Molkentin JD, Lu JR, Antos CL et al. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* 1998 April 17;93(2):215-28.

(12) Severs NJ, Coppen SR, Dupont E, Yeh HI, Ko YS, Matsushita T. Gap junction alterations in human cardiac disease. *Cardiovasc Res* 2004 May 1;62(2):368-77.

(13) van Rijen HV, Eckardt D, Degen J et al. Slow conduction and enhanced anisotropy increase the propensity for ventricular tachyarrhythmias in adult mice with induced deletion of connexin43. *Circulation* 2004 March 2;109(8):1048-55.

(14) van Veen TA, Stein M, Royer A et al. Impaired impulse propagation in Scn5a-knockout mice: combined contribution of excitability, connexin expression, and tissue architecture in relation to aging. *Circulation* 2005 September 27;112(13):1927-35.

(15) Jansen JA, Noorman M, Musa H et al. Reduced heterogeneous expression of Cx43 results in decreased Nav1.5 expression and reduced sodium current that accounts for arrhythmia vulnerability in conditional Cx43 knockout mice. *Heart Rhythm* 2012 April;9(4):600-7.

(16) Jansen JA, van Veen TA, de Jong S et al. Reduced Cx43 expression triggers increased fibrosis due to enhanced fibroblast activity. *Circ Arrhythm Electrophysiol* 2012 April;5(2):380-90.

(17) Ling H, Zhang T, Pereira L et al. Requirement for Ca^{2+}/calmodulin-dependent kinase II in the transition from pressure overload-induced cardiac hypertrophy to heart failure in mice. *J Clin Invest* 2009 May;119(5):1230-40.

(18) Sedej S, Heinzel FR, Walther S et al. Na^{+}-dependent SR Ca^{2+}overload induces arrhythmogenic events in mouse cardiomyocytes with a human CPVT mutation. *Cardiovasc Res* 2010 July 1;87(1):50-9.

(19) Karczewski P, Kuschel M, Baltas LG, Bartel S, Krause EG. Site-specific phosphorylation of a phospholamban peptide by cyclic nucleotide- and Ca2+/calmodulin-dependent protein kinases of cardiac sarcoplasmic reticulum. *Basic Res Cardiol* 1997;92 Suppl 1:37-43.

(20) Blaich A, Welling A, Fischer S et al. Facilitation of murine cardiac L-type Ca(v)1.2 channel is modulated by calmodulin kinase II-dependent phosphorylation of S1512 and S1570. *Proc Natl Acad Sci U S A* 2010 June 1;107(22):10285-9.

(21) Ai X, Curran JW, Shannon TR, Bers DM, Pogwizd SM. Ca2+/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca2+ leak in heart failure. *Circ Res* 2005 December 9;97(12):1314-22.

(22) Antoons G, Sipido KR. Targeting calcium handling in arrhythmias. *Europace* 2008 December;10(12):1364-9.

(23) MacDonnell SM, Weisser-Thomas J, Kubo H et al. CaMKII negatively regulates calcineurin-NFAT signaling in cardiac myocytes. *Circ Res* 2009 August 14;105(4):316-25.

(24) Xiao L, Coutu P, Villeneuve LR et al. Mechanisms underlying rate-dependent remodeling of transient outward potassium current in canine ventricular myocytes. *Circ Res* 2008 September 26;103(7):733-42.

(25) Hoch B, Meyer R, Hetzer R, Krause EG, Karczewski P. Identification and expression of delta-isoforms of the multifunctional Ca2+/calmodulin-dependent protein kinase in failing and nonfailing human myocardium. *Circ Res* 1999 April 2;84(6):713-21.

(26) Maier LS, Zhang T, Chen L, DeSantiago J, Brown JH, Bers DM. Transgenic CaMKIIdeltaC overexpression uniquely alters cardiac myocyte Ca2+ handling: reduced SR Ca2+ load and activated SR Ca2+ release. *Circ Res* 2003 May 2;92(8):904-11.

(27) Cruciani V, Kaalhus O, Mikalsen SO. Phosphatases involved in modulation of gap junctional intercellular communication and dephosphorylation of connexin43 in hamster fibroblasts: 2B or not 2B? *Exp Cell Res* 1999 November 1;252(2):449-63.

(28) Li J, Patel VV, Kostetskii I et al. Cardiac-specific loss of N-cadherin leads to alteration in connexins with conduction slowing and arrhythmogenesis. *Circ Res* 2005 September 2;97(5):474-81.

(29) Kaplan SR, Gard JJ, Carvajal-Huerta L, Ruiz-Cabezas JC, Thiene G, Saffitz JE. Structural and molecular pathology of the heart in Carvajal syndrome. *Cardiovasc Pathol* 2004 January;13(1):26-32.

(30) Kaplan SR, Gard JJ, Protonotarios N et al. Remodeling of myocyte gap junctions in arrhythmogenic right ventricular cardiomyopathy due to a deletion in plakoglobin (Naxos disease). *Heart Rhythm* 2004 May;1(1):3-11.

(31) Oxford EM, Musa H, Maass K, Coombs W, Taffet SM, Delmar M. Connexin43 remodeling caused by inhibition of plakophilin-2 expression in cardiac cells. *Circ Res* 2007 September 28;101(7):703-11.

(32) Bruce AF, Rothery S, Dupont E, Severs NJ. Gap junction remodelling in human heart failure is associated with increased interaction of connexin43 with ZO-1. *Cardiovasc Res* 2008 March 1;77(4):757-65.

(33) Anderson C, Catoe H, Werner R. MIR-206 regulates connexin43 expression during skeletal muscle development. *Nucleic Acids Res* 2006;34(20):5863-71.

(34) van Rooij E, Sutherland LB, Liu N et al. A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. *Proc Natl Acad Sci U S A* 2006 November 28;103(48):18255-60.

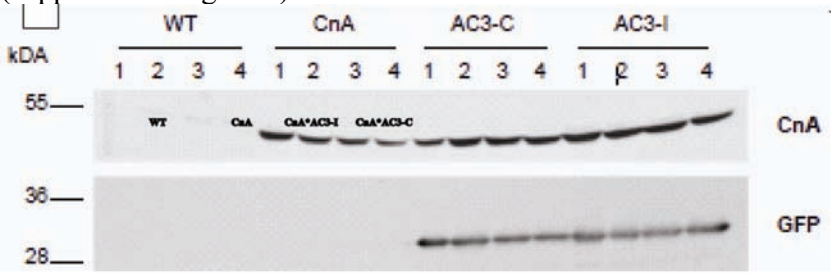
(35) da Costa Martins PA, Salic K, Gladka MM et al. MicroRNA-199b targets the nuclear kinase Dyrk1a in an auto-amplification loop promoting calcineurin/NFAT signalling. *Nat Cell Biol* 2010 December;12(12):1220-7.

Supplemental materials

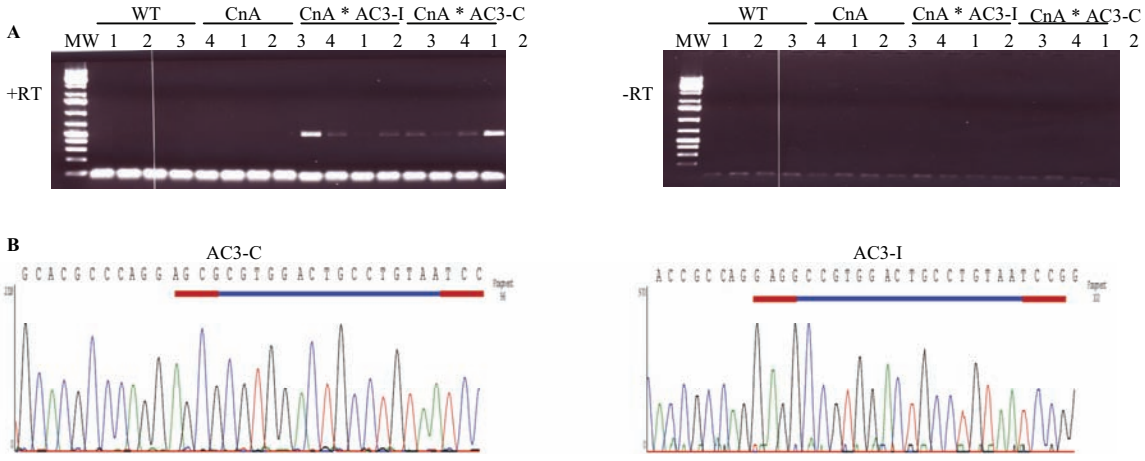
In vitro internal controls:

To determine the phenotype of the hearts, western blotting was performed against CnA and GFP (supplemental figure 1). CnA and CnA*AC3-I/C mice expressed much higher levels of CnA than WT mice, as expected.

Similarly, only the mice that also express AC3-I or AC3-C expressed the GFP tag. To discriminate between AC3-I and AC3-C, more tools were necessary, as these groups both expressed the same GFP tag. To assess this difference, RT-PCR was performed with specific primers and the obtained products sequenced to discriminate between AC3-C and AC3-I (supplemental figure 2).



Supplemental figure 1: CnA and GFP Western blots.
WT mice do not express the constitutively active CnA, and only AC3-C/I mice also express the GFP tag.

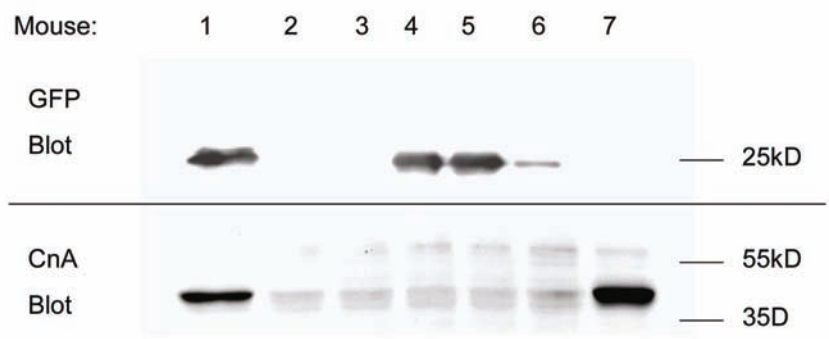


Supplemental figure 2: RT-PCR on samples from AC3-I or AC3-C hearts.
A) RT-PCR results from WT, CnA, CnA*AC3-C and CnA*AC3-I ventricular RNA samples using specific primers against AC3-C and AC3-I. +RT reaction was performed in the presence of reverse transcriptase enzyme; as a control the reaction was also performed without reverse transcriptase enzyme (-RT).
B) A representative sequence analysis is shown for AC3-C and AC3-I. MW, molecular weight marker.

In vivo internal controls

The Langendorff perfusion pilot experiments were performed in offspring from CnA males and AC3-I females. As the genotypes of these litters were not known prior to the Langendorff experiments, they were thus performed blind.

Afterwards, Western blotting was performed for CnA and GFP. The CnA blot should be able to discriminate between WT or AC3-I (low CnA) and CnA or CnA*AC3-I (high CnA). The presence of GFP should then be able to discern the mice with or without AC3-I. Combination of these blots was able to categorize every heart (Supplemental figure 3).



Mouse #	CnA	GFP	Conclusion:
1	+	+	CnA*AC3-I
2	-	-	WT
3	-	-	WT
4	-	+	AC3-I
5	-	+	AC3-I
6	-	-	WT
7	+	-	CnA

Supplemental figure 3: Categorization of mice hearts used for Langendorff perfusion experiments. Upper panel: GFP and CnA blots. Below: Characterization of hearts based on above blots.

Primers

The used primers can be found below:

	SEQUENCE:	TARGET:
Primer 1	TGCTTACTTCAATGGCTGCT	Cx43 exon 2
Primer 2	GGAGGAATTGTTTCTGTCACC	Cx43 exon 2
Primer 3	CGTGCCCTGTTGTATCCTGACA	Scn5a 3′-UTR
Primer 4	AGAACTCTGCCGTGTGGTTCCT	Scn5a 3′-UTR
Primer 7	GCCGTATTCATTGTCATACCAGGA	GAPDH
Primer 8	CCTGCCAAGTATGATGACATCAAG	GAPDH
Primer 9	AGGCGATTGATCCCAAGTTGTCG	CnA α
Primer 10	TGCCCTCCTTCATGAGATGTGCTT	CnA α

Supplemental table 1: sequences and targets of used primers.

Chapter 7:

Verapamil as an antiarrhythmic agent in congestive heart failure: hopping from rabbit to human?

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Abstract

Repolarization-dependent cardiac arrhythmias only arise in hearts facing multiple 'challenges' affecting its so-called repolarization reserve. Congestive heart failure (CHF) is one such challenge frequently observed in humans and is accompanied by altered calcium handling within the contractile heart cell. This raises the question as to whether or not the well-known calcium channel antagonist verapamil acts as an antiarrhythmic drug in this setting, as seen in arrhythmia models without CHF. According to the study of Milberg et al. in this issue of BJP, the answer is yes. The results of this study, using a rabbit CHF model, raise important questions. First, given that the model combines CHF with a number of other interventions that predispose towards arrhythmia, will similar conclusions be reached in a setting where CHF is a more prominent proarrhythmic challenge; second, what is the extent to which other effects of calcium channel block would limit the clinical viability of this pharmacological approach in CHF? In vivo studies in large animal CHF models are now required to further explore this interesting, but complex, approach to the treatment of arrhythmia.

Abbreviations
CHF: congestive heart failure
EAD: early after depolarization
ICa-L: L-type calcium current
IKr: rapid delayed rectifier Channel
TdP: torsade de pointes

In cardiac ventricular myocytes, an imbalance between inward and outward currents may prolong action potential duration. This makes the heart vulnerable to the occurrence of so-called torsade de pointes (TdP) arrhythmias, which are life-threatening ventricular tachycardias that create rapid fluctuations of QRS complexes around the isoelectric line on the human ECG. In patients, there are several independent risk factors or 'challenges' for TdP arrhythmias, including hypokalaemia, bradycardia, genetic and drug-induced long QT syndromes and chronic congestive heart failure (CHF) (Roden, 2004). A single 'challenge' to cardiac ventricular repolarization, for example, the reduction of a single membrane ion current, usually does not result in repolarization-dependent arrhythmias. Apparently, the heart has a reserve, commonly referred to as 'repolarization reserve' (Varró and Baczkó, 2011), and multiple challenges are therefore usually required in order to provoke arrhythmia. Often, QT-prolonging drugs associated with TdP arrhythmias are the final challenge that exceeds the reserve, resulting in proarrhythmia. Quantification of the repolarization reserve, however, remains difficult. Although a number of surrogate parameters have been suggested (Thomsen *et al*., 2006), such as temporal or spatial dispersion of action potential duration, optimal quantification of repolarization reserve still requires testing of susceptibility to arrhythmias, where the cumulative severity of the challenges required to exceed the reserve then provides an estimation of the reserve. Interestingly, some drugs, including those that block the inward L-type calcium current ($I_{Ca\text{-}L}$), have been shown to be effective against drug-induced arrhythmias, by counteracting one or more of the predisposing challenges (Oros *et al*., 2010).

Only a few experimental large animal models mimicking CHF have been developed. Currently, the efficacy in which $I_{Ca\text{-}L}$ inhibition prevents or suppresses early after depolarizations (EAD) and polymorphic ventricular tachycardia in CHF is not clear and difficult to predict since calcium-handling disturbances are apparent in this disease (Janse, 2004). Moreover, in a setting of CHF, this apparent simple antiarrhythmic approach has to deal with conflicting imperatives such as antiarrhythmic action versus haemodynamic tolerance. In this issue of the *BJP*, Milberg *et al*. (2012a) report the outcome of $I_{Ca\text{-}L}$ block by verapamil, a well established antiarrhythmic compound, on arrhythmic end points in a rabbit model of non-ischemic CHF with long-QT characteristics (Milberg *et al*., 2012a). CHF was generated by continuous right ventricular rapid pacing and, subsequently, Langendorff-perfused sham and CHF hearts were subjected to a number of additional challenges in order to provoke arrhythmias: bradycardia, ectopic ventricular activation, severe hypokalaemia and erythromycin-mediated I_{Kr} block. Repolarization was prolonged to some extent in CHF but spatial dispersion was not affected at baseline. Only after I_{Kr}block, especially transmural dispersion was increased to a larger extent in CHF. Arrhythmias were observed, but their number in hearts from sham animals (four of 11 hearts; 36%) was not significantly different from rabbit hearts with CHF (eight of 11; 73%; P = NS). Unfortunately, surrogate parameters were only reported for normokalemic circumstances, when the repolarization reserve was challenged less severely and thus could not directly be associated with the arrhythmic end point. Remarkably, the findings of the same group published recently (Frommeyer *et al*., 2011), in which the rabbit hearts were used to analyse the proarrhythmic effect of the I_{Kr} blocker sotalol were in favour of the CHF model used here . In this CHF group, sotalol induced EADs (as estimated from monophasic action potential morphology) and TdP in 16 of 18 (89%) hearts compared with seven of 14 (50%) hearts in the sham group. When we solely compare arrhythmia incidence based on these numbers, a P-value of 0.023 is obtained (two-tailed Fisher exact test). However, in both studies, and yet another [seven of 14 (50%) Milberg *et al*., 2012b], the pronounced incidence of arrhythmias in the sham hearts represents a potential limitation. Nevertheless, verapamil was demonstrated to be an efficient

antiarrhythmic drug in this setting, and importantly, we may thus conclude that effectiveness of $I_{Ca\text{-}L}$ block as antiarrhythmic treatment persists in an isolated rabbit heart model where CHF is added. The next hurdle will be to reach similar conclusions in an *in vivo* model where CHF is a more prominent proarrhythmic factor.

Mechanisms of the antiarrhythmic potential of verapamil against repolarization-dependent arrhythmias have been ascribed to shortening of the QT interval and decreases in beat-to-beat variability of action potential duration (Oros *et al*., 2010; Bourgonje *et al*., 2011), and nowMilberg *et al*. (2012a) show that it counteracts spatial dispersion in a CHF heart too. Promising as it seems, verapamil is contraindicated in CHF, especially in cases with severe systolic dysfunction and reduced fractional shortening (Chew *et al*., 1981). As verapamil inhibits the systolic calcium flux and, consequently, contractility, it is negatively inotropic, and this makes verapamil probably a poor choice in the clinic; certainly, when considering that the concentration used by Milberg *et al*. (0.75 μM) was unable to suppress arrhythmias completely. In the *in vivo* complete atrial-ventricular block dog model, verapamil plasma levels of around 0.5 μM clearly were antiarrhythmic but also lowered left ventricular pressure (Oros *et al*., 2010). Upon titrating verapamil, antiarrhythmic activity could not be observed without a drop in left ventricular pressure (Bourgonje *et al*., 2011). Other calcium channel antagonists might be a better option, however, and the authors themselves advocate second-generation $I_{Ca\text{-}L}$ blockers. Take, for instance, nifedipine that more strongly affects smooth than striated muscle (Millard *et al*., 1983), where lowering peripheral resistance would compensate for negative inotropy. This may still have a major drawback because, in order to preserve blood pressure where contractility is reduced and vessels are dilated, heart rate must increase, which is also unfavourable for an already weakened heart. Obviously, it would be hard to predict the individual effects on vasodilatation and cardiac contractility, and where they would counterbalance each other in a haemodynamically challenged heart under neurohumoral influence. This should be approached experimentally. Furthermore, while inhibiting systolic calcium may be worrisome, in the case of diastolic dysfunction, calcium channel antagonism might be beneficial by improving coronary flow and muscle relaxation. As answering these questions is beyond the opportunities offered by the model of Milberg *et al*. (2012a), other models should be employed to address these intriguing possibilities.

In conclusion, the study of Milberg *et al*. (2012a) demonstrates the efficacy of verapamil as an antiarrhythmic agent in the setting of CHF and provides basic science insights into its mechanism of action of reducing spatial dispersion. Further studies are required to pinpoint the contribution of CHF to arrhythmogenesis in this model, to recapitulate the findings in models where CHF is a more pronounced proarrhythmic challenge and to validate the antiarrhythmic efficacy and demonstrate clinical feasibility, of $I_{Ca\text{-}L}$ block in *in vivo* models of CHF.

Acknowledgements
We thank Rebecca Burton, Department of Physiology, Anatomy, and Genetics, University of Oxford, for careful reading of the manuscript. This work received funding from the European Community's Seventh Framework Programme FP7/2007–2013 under grant agreement no. HEALTH-F2-2009-241526 EUTrigTreat and by a grant from the Fondation Leducq and the Alliance for Calmodulin Kinase II Signaling in Heart Disease.

References

Bourgonje VJ, Vos MA, Ozdemir S, Doisne N, Acsai K, Bito V *et al*. (2011). SEA0400 provides anti-arrhythmic actions against dofetilide-induced torsade de pointes (TdP) without a negative inotropic effect. Heart Rhythm 8: S330–S331.

Chew CY, Hecht HS, Collett JT, McAllister RG, Singh BN (1981). Influence of severity of ventricular dysfunction on hemodynamic responses to intravenously administered verapamil in ischemic heart disease. Am J Cardiol 47: 917–922.

Frommeyer G, Milberg P, Witte P, Stypmann J, Koopmann M, Lücke M *et al*. (2011). A new mechanism preventing proarrhythmia in chronic heart failure: rapid phase-III repolarization explains the low proarrhythmic potential of amiodarone in contrast to sotalol in a model of pacing-induced heart failure. Eur J Heart Fail 13: 1060–1069.

Janse MJ (2004). Electrophysiological changes in heart failure and their relationship to arrhythmogenesis. Cardiovasc Res 61: 208–217.

Milberg P, Fink M, Pott C, Frommeyer G, Biertz J, Osada N *et al*. (2012a). ICa block leads to suppression of early afterdepolarizations and reduction of transmural dispersion of repolarization in a whole heart model of chronic heart failure. Br J Pharmacol 166: 557–568.

Milberg P, Frommeyer G, Uphaus T, Kaiser D, Osada N, Breithardt G *et al*. (2012b). Electrophysiologic profile of dronedarone on the ventricular level: beneficial effect on post repolarization refractoriness in the presence of rapid phase-IIIrepolarization. J Cardiovasc Pharmacol 59: 92–100.

Millard RW, Grupp G, Grupp IL, DiSalvo J, DePover A, Schwartz A (1983). Chronotropic, inotropic, and vasodilator actions of diltiazem, nifedipine, and verapamil. A comparative study of physiological responses and membrane receptor activity. Circ Res 52: I29–I39.

Oros A, Houtman MJ, Neco P, Gomez AM, Rajamani S, Oosterhoff P *et al*. (2010). Robust anti-arrhythmic efficacy of verapamil and flunarizine against dofetilide-induced TdP arrhythmias is based upon a shared and a different mode of action. Br J Pharmacol 161: 162–175.

Roden DM (2004). Drug-induced prolongation of the QT interval. N Engl J Med 350: 1013–1022.

Thomsen MB, Matz J, Volders PG, Vos MA (2006). Assessing the proarrhythmic potential of drugs: current status of models and surrogate parameters of torsades de pointes arrhythmias. Pharmacol Ther 112: 150–170.

Varró A, Baczkó I (2011). Cardiac ventricular repolarization reserve: a principle for understanding drug-related proarrhythmic risk. Br J Pharmacol 164: 14–36.

Chapter 8:

Combined Na^+/Ca^{2+} exchanger and L-type calcium channel block as a potential strategy to suppress arrhythmias and maintain ventricular function

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Abstract

Background:
L-type calcium channel (LTCC) and Na^{+}/Ca^{2+} exchanger (NCX) have been implicated in repolarization-dependent arrhythmias, but also modulate calcium and contractility. While LTCC inhibition is negative inotropic, NCX inhibition has the opposite effect. Combined block may therefore offer an advantage for hemodynamics and antiarrhythmic efficiency, particularly in diseased hearts.
In a model of proarrhythmia, the dog with chronic atrioventricular block (CAVB), we investigated if combined inhibition of NCX and LTCC with SEA-0400 is effective against dofetilide-induced Torsade de Pointes arrhythmias (TdP), while maintaining calcium homeostasis and hemodynamics.

Methods & Results:
Left ventricular pressure (LVP) and ECG were monitored during infusion of SEA-0400 and verapamil in anesthetized dogs. Different doses were tested against dofetilide-induced TdP in CAVB dogs. In ventricular myocytes, effects of SEA-0400 were tested on action potentials (AP), calcium transients, and early afterdepolarizations (EAD).
In cardiomyocytes, SEA-0400 (1 μM) blocked 66±3% of outward NCX, 50±2% of inward NCX, and 33±9% of LTCC current. SEA-0400 had no effect on systolic calcium, but slowed relaxation despite AP shortening, and increased diastolic calcium. SEA-0400 stabilized dofetilide-induced lability of repolarization and suppressed EADs. In vivo, SEA-0400 (0.4 and 0.8 mg/kg) had no effect on LVP, and suppressed dofetilide-induced TdPs dose-dependently. Verapamil (0.3 mg/kg) also inhibited TdP, but caused a 15±8% drop of LVP. A lower dose of verapamil without effects on LVP (0.06 mg/kg) was not anti-arrhythmic.

Conclusion:
In CAVB dogs, SEA-0400 treatment is effective against TdP. Unlike specific inhibition of LTCC, combined NCX and LTCC inhibition has no negative effects on cardiac hemodynamics.

Abbreviations

AP(D): action potential (duration)
CAVB: chronic atrioventricular block
EAD: early afterdepolarization
LTCC: L-type calcium channel
LV(P): Left ventricular (pressure)
NCX: sodium/calcium exchanger
STV: short-term variability of repolarization
TdP: Torsades de Pointes arrhythmia

Introduction

The 2006 guidelines for treatment of life-threatening ventricular arrhythmias advocate device therapy for high-risk patients over drug therapy as primary strategy. Unsuccesful anti-arrhythmic drug trials, together with positive ICD trials, are at the basis of this approach. Despite succesful device therapy, an unmet need for efficient drug therapy exists, for cost reasons as well as shock reduction and improved quality of life. Novel targets offer opportunities to revisit drug therapy (Billman, GE. Novel Therapeutic targets for antiarrhythmic drugs. 2010. John Wiley & Sons, Inc.).
Remodeling in failure or compensated hypertrophy is often accompanied by AP prolongation [1] and susceptibility for repolarization-dependent arrhythmias. Calcium antagonists, like the LTCC blocker verapamil, and magnesium sulphate [2], can effectively treat TdP, in experimental and clinical settings[2-4], but are negative hemodynamic [5;6], and therefore contraindicated in heart failure patients.
In this study, we explore if combined LTCC and NCX block by SEA-0400 is a potential anti-arrhythmic strategy against EADs/TdPs, which overcomes the negative inotropic effects of selective LTCC block, by limiting Ca^{2+} efflux via NCX. Also, the NCX current has been implicated in EAD formation [7;8] and thus inhibition of NCX may add to the anti-arrhythmic effect. Importantly, in the normal heart, SEA-0400 has no negative effects on $[Ca^{2+}]_i$ [9;10], or even positive effects [9-11]. The net effect of SEA-0400 in disease could be different because of disturbed Ca^{2+} and Na^{+} balances[12].
The anti-arrhythmic potential of SEA-0400 is not completely established. In LQT models, data are contradictory, with positive[7;13;14] and negative results[15;16]. Recently, SEA-0400 was reported to be anti-arrhythmic in failing rabbit hearts[17].
The present study is the first to explore the combination of anti-arrhythmic efficacy with the presumed neutral hemodynamic effects of SEA-0400, in a model with high TdP susceptibility, the CAVB dog [18]. Common calcium antagonists, despite anti-arrhythmic potential, have limited usefulness due to negative inotropic effects. SEA-0400 might be able to maintain hemodynamics and thus open calcium channel block to wider clinical application. Its effects will be compared to the classical calcium inhibitor verapamil, which is very effective in abolishing TdP [3].

Materials & Methods

In vivo experiments
37 experiments were performed in 15 dogs.
In a first set (n=16) , hemodynamic effects were determined by measuring LVP during infusion of verapamil or SEA-0400.
In the second set (n=12), arrhythmias were induced using dofetilide, after which verapamil or SEA-0400 was infused as an anti-arrhythmic.
For additional details, see supplemental data.

Cellular experiments
Experiments were performed at 37^{o}C in myocytes, enzymatically isolated from the LV midmyocardial layer of CAVB hearts [19].

APs and membrane currents were recorded in the whole-cell patch clamp mode, with simultaneous recording of Ca^{2+} signals in epifluorescence mode. [20]. See supplemental data for protocols and solutions.

SEA-0400 plasma concentrations
Blood samples were collected through a venous catheter every 5 min during experiment. Heparin-treated samples were centrifuged at 1300rcf and stored at -80 ^{o}C for further analysis. Concentrations of SEA-0400 were determined by high-performance liquid chromatography

Statistics
See supplemental data.

Results

Quantification of NCX and LTCC block by SEA-0400
We quantified the effect of 1 μM SEA-0400 on NCX-mediated currents (I_{NCX}), and inward Ca^{2+} current mediated through LTCC (I_{CaL}) in CAVB myocytes.
I_{NCX} was measured as the Ni^{2+} sensitive current during voltage ramps, at constant $[Na^{+}]_i$ (10 mM) and $[Ca^{2+}]_i$ (~ 100 nM free Ca^{2+}) (Fig 1A). SEA-0400 inhibited 66±3% of outward, and 50±2% of inward I_{NCX} (n=5, Fig 1B).
I_{CaL} was measured during a depolarizing step to +10 mV (low SR Ca^{2+} load, 0.1 Hz repletion rate, Fig 1C). I_{CaL} block by SEA-0400 was 33±9% (n=6, Fig 1D). Note the reduced inward tail current on repolarization (Fig 1E,a) despite higher $[Ca^{2+}]_i$ levels (Fig 1E,b,c), which reflects forward NCX block (n=4, Fig 1E,d). Despite partial LTCC block, peak and amplitude of the Ca^{2+} transient were increased.

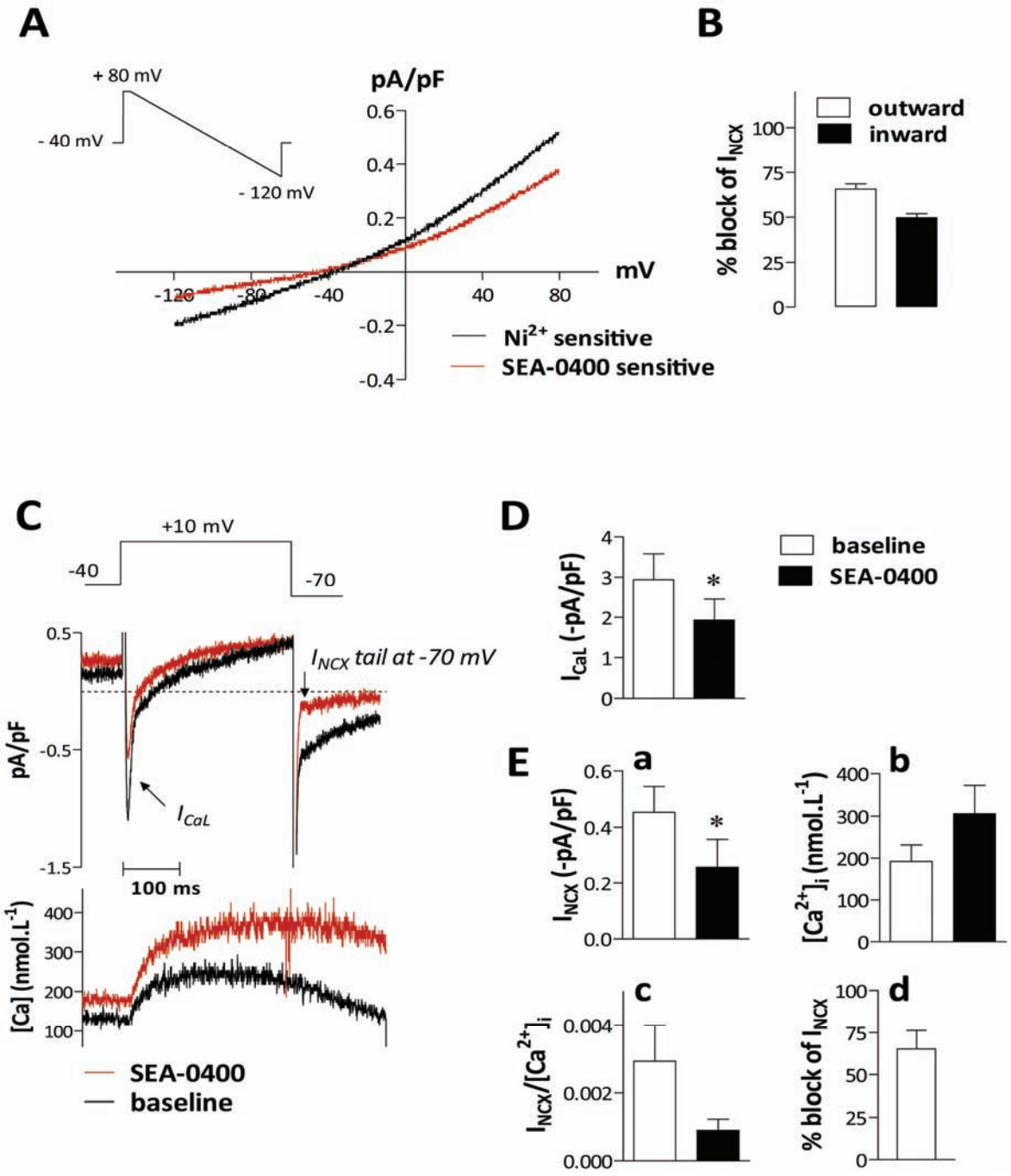

Figure 1. Block of NCX and I_{CaL} by SEA-0400

A, NCX currents sensitive to 5mM Ni^{2+} (total I_{NCX}, black) and 1µM SEA-0400 (red) during repolarizing ramps (step from -40mV to +80mV, descending ramp to -120mV in 2s; averaged current traces of 5 cells). **B**, Fraction of total I_{NCX} blocked by 1µM SEA-0400. Outward and inward I_{NCX} were calculated at 60mV on either side of the reversal potential.

C, Example of I_{CaL} and Ca^{2+} transients recorded during a depolarizing step to +10mV before and after 1µM SEA-0400. I_{Na} was inactivated by a prepulse to -40mV, and currents were recorded in K^+ and EGTA free solutions.

D, I_{CaL} amplitude under baseline and with SEA-0400 (n=6).

E, Pooled data of NCX tail current density (**a**) and $[Ca^{2+}]_i$ (**b**) on repolarization to -70mV; **c**, I_{NCX} normalized to $[Ca^{2+}]$; **d**, Percentage of NCX tail current block at -70mV (n=4, *, P<0.05).

SEA-0400 effects on AP and $[Ca^{2+}]_i$

The effect of SEA-0400 on $[Ca^{2+}]_i$ and APs is illustrated in Fig 2A. Red traces were recorded when wash-in of SEA-0400 had reached steady-state. SEA-0400 had no effects on peak $[Ca^{2+}]_i$, but increased diastolic $[Ca^{2+}]_i$, slowed relaxation, and shortened APD (Fig 2). We also recorded Ca^{2+} transients and APs during wash-out (blue traces). We have previously observed that LTCC block by SEA-0400 was rapidly reversible upon washout, while NCX block was not [12]. The removal of LTCC inhibition had pronounced effects on the Ca^{2+} transients during wash-out. There was a twofold increase of peak $[Ca^{2+}]_i$, further impairment of relaxation, and a larger increase of diastolic $[Ca^{2+}]_i$. APs re-lengthened (n=5, Fig 2B).

These data illustrate that partial NCX block causes a net gain of Ca^{2+}, which is counterbalanced by reduced I_{CaL} during combined block. The changes in AP may contribute to these changes in Ca^{2+} balance.

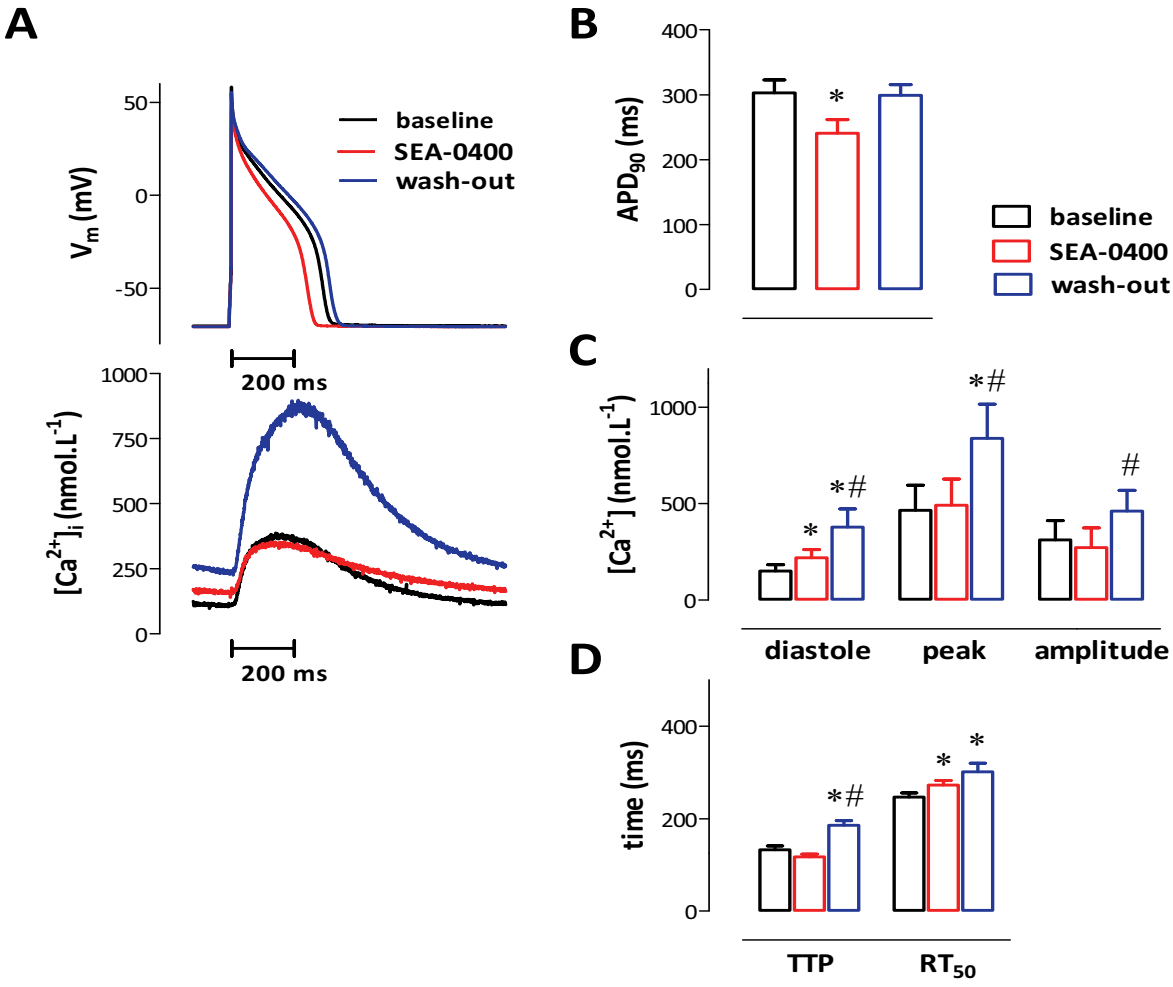

Figure 2. Effects of SEA-0400 on AP and Ca^{2+} transients

A, Example of APs (top) and Ca^{2+}-transients (bottom), under baseline (black), 1µM SEA-0400 (red) and during wash-out (blue), in a CAVB cell at 1Hz.

B, Pooled data of APD_{90}.

C, diastolic and peak $[Ca^{2+}]_i$, and Ca transient amplitude (peak-baseline).

D, Kinetics of Ca^{2+} transient; ttp, time-to-peak; RT_{50}, time to half maximal relaxation. *, P<0.05 vs baseline, # vs SEA-0400. n= 5 cells.

Effects of SEA-0400 on EADs and APD

Fig 3A shows a typical experiment testing effects of SEA-0400 against dofetilide-induced EADs in a CAVB cell, and Fig 3B shows beat-to-beat changes in APD and short-term variability of repolarization (STV, red line), a marker of proarrhythmia. Typically, dofetilide prolonged AP and increased STV. SEA-0400 was applied after the first EAD appeared. In all cells (n=11), SEA-0400 suppressed EADs and restored STV (Fig 3C). In Fig 3D, we plotted individual data of STV in function of APD. This revealed a positive relation between STV and AP prolongation in the presence of dofetilide; SEA-0400 caused a downward shift of this curve. In a subset of cells (n=5), we monitored changes in $[Ca^{2+}]_i$ during wash-in of dofetilide and SEA-0400 (Fig 3E). Dofetilide alone increased peak and amplitude of the Ca^{2+} transient.. SEA-0400 caused a further increase of diastolic, but not peak $[Ca^{2+}]_i$; the amplitude was comparable with baseline.

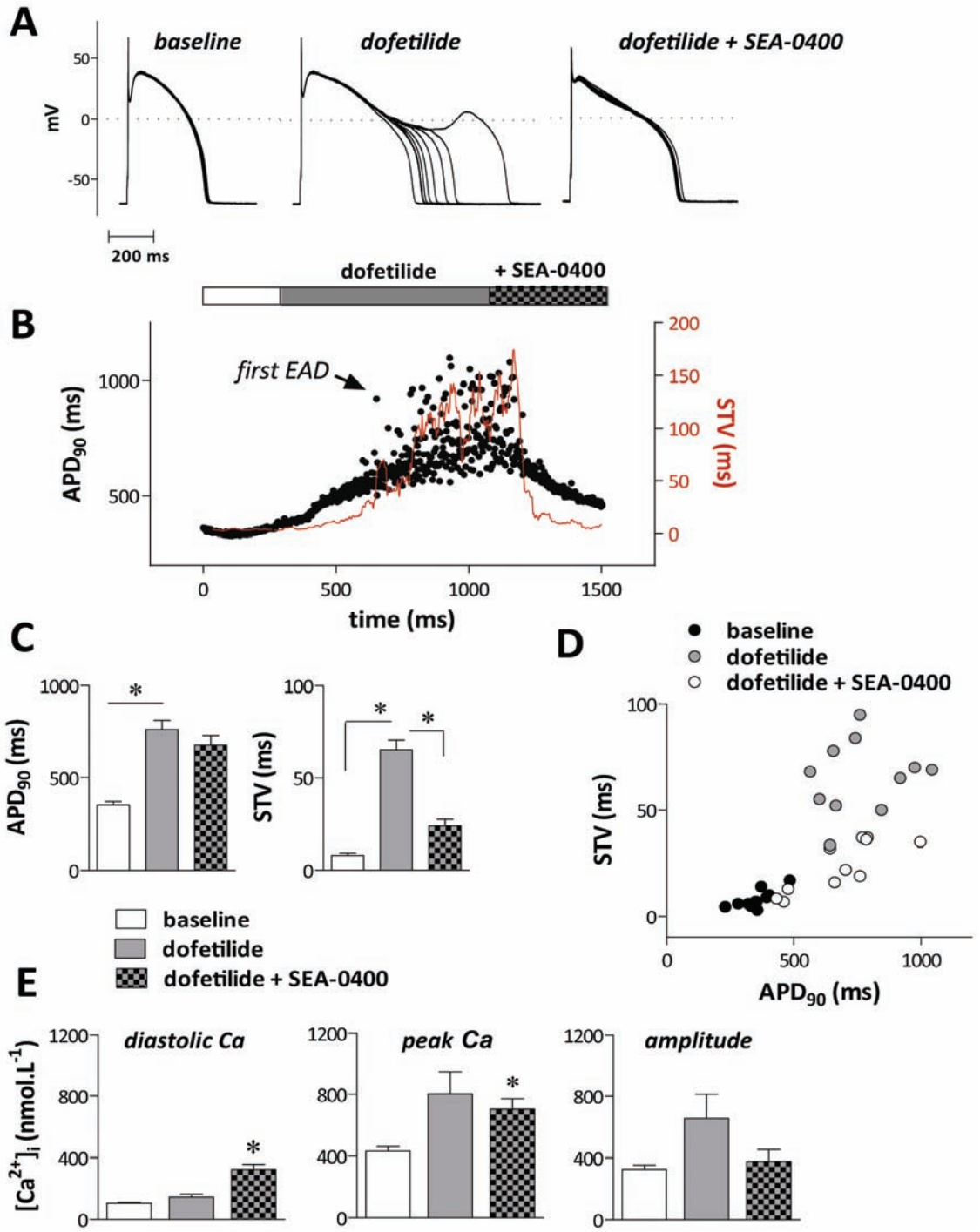

Figure 3. Anti-arrhythmic effects of SEA0400 against EADs

A, Recordings of APs at 0.5Hz in a CAVB cell showing dofetilide-induced EADs and suppression by SEA-0400 (1μM).

B, Time-dependent changes in APD_{90} (symbols), STV (red line) and EADs during wash-in of dofetilide and SEA-0400.

C, Pooled data of APD_{90} (left panel) and STV (right panel) of 11 cells. For dofetilide, APD and STV values are an average of 30 successive beats prior to the first EAD (* indicates $P<0.05$).

D, Individual data of STV in function of APD_{90}.

E. Parameters of Ca^{2+} transients during the different treatments; averaged data of 5 cells (* $P<0.05$ vs baseline).

SEA-0400 preserves LVP, while verapamil is negative inotropic.
Prior to anti-arrhythmic testing, we examined baseline effects of SEA-0400 in anesthetized sinus rhythm and CAVB dogs. CAVB dogs have lower heart rates, prolonged QT and higher LVP. SEA-0400 was administered in cumulative doses over a 5 min infusion period, to a final dose of 0.4 mg/kg or 0.8 mg/kg. This resulted in peak plasma concentrations of resp. 5±1 μM, and 11±2 μM at 5 min after the start of infusion, and 1.5±0.3 μM, and 4.2±0.5 μM at 10 min (n=3-7). Neither SEA-0400 dose had an effect on heart rate, QT-time, STV-QT, nor diastolic and maximal LVP (Table 1).
In Fig 4, the relative change of LVP during infusion of SEA-0400 was compared with verapamil. At a cumulative dose of 0.3 mg/kg, verapamil caused a 15% drop in systolic LVP, with no effects on HR, QT or baseline STV (Table 1). Based on this dose-pressure response (Fig 4), two dosages of verapamil were chosen for anti-arrhythmic testing: a hemodynamically neutral (0.06 mg/kg) and a negative inotropic dose (0.3 mg/kg). Absolute changes in LVP can be found in table 1, divided between SR and CAVB dogs, since CAVB dogs are known to have a higher baseline LVP.

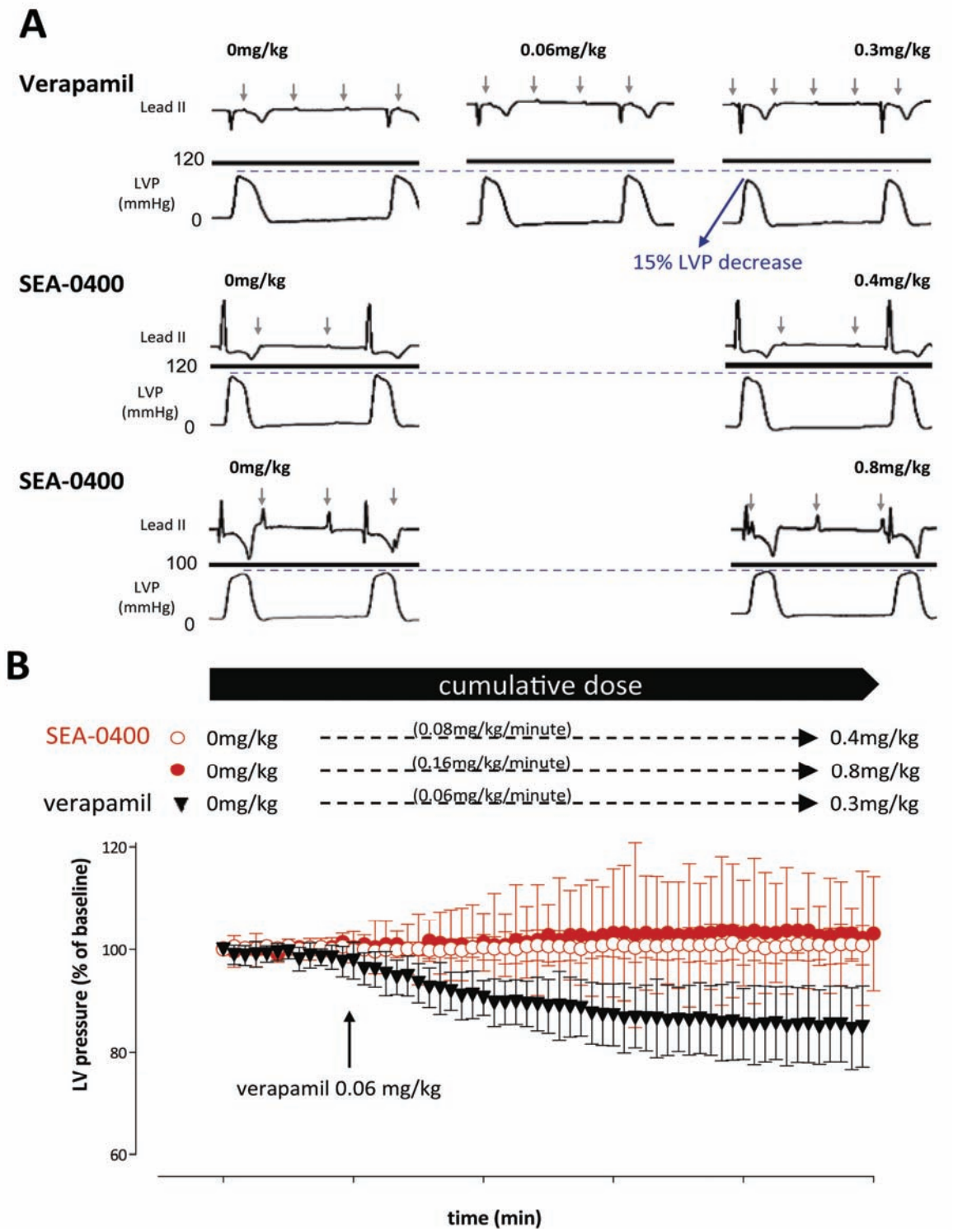

Figure 4. Relative pressure changes during verapamil or SEA-0400 infusion

A, Examples of left ventricular pressure curves during verapamil of SEA-0400 treatment. Lead II was inserted for reference. P-waves are denoted with grey arrows. Note that, as these are CAVB dogs, P-waves and QRS complexes do not correlate. The blue dashed lines indicate the maximum pressure of the curves at baseline.

B, Relative changes of systolic LVP during cumulative infusion of (▼) verapamil, 0.3 mg/kg/5min (N=6); (O) SEA-0400, 0.4 mg/kg/5min (N=6); (●) SEA-0400 0.8 mg/kg/5min (●, N=4). Arrow depicts the highest cumulative dose of verapamil without effect on pressure ('hemodynamic neutral' dose, 0.06 mg/kg).

SEA-0400 suppresses TdP while preserving LVP

Dofetilide induced TdP in 6/9 dogs (67%) (Fig. 5A). TdPs were suppressed by verapamil and SEA-0400 (Fig. 5B and C). Dofetilide caused QT-prolongation and increased STV-QT. Subsequent administration of a low dose of verapamil did not suppress TdPs (11± 6 episodes per 5 min vs 12±7), while the higher dose was completely effective (0 TdPs). This was associated with reduced STV-QT, without shortening QT-time (5A, lower graph). These parameters could not be determined at the low dose of verapamil, because arrhythmias interfered with measurements.

SEA-0400 was anti-arrhythmic (Fig 5C) with a dose-dependent effect: 0.4 mg/kg partially suppressed (from 7±4 to 3±4 episodes per 5 min), and 0.8 mg/kg completely abolished TdPs. The partial anti-arrhythmic effect of 0.4 mg/kg SEA-0400 did not prevent occurrence of extra beats, which excluded reliable STV-QT measurements. At 0.8 mg/kg, SEA-0400 tended to reduce STV-QT, and had no effect on dofetilide-induced QT prolongation.

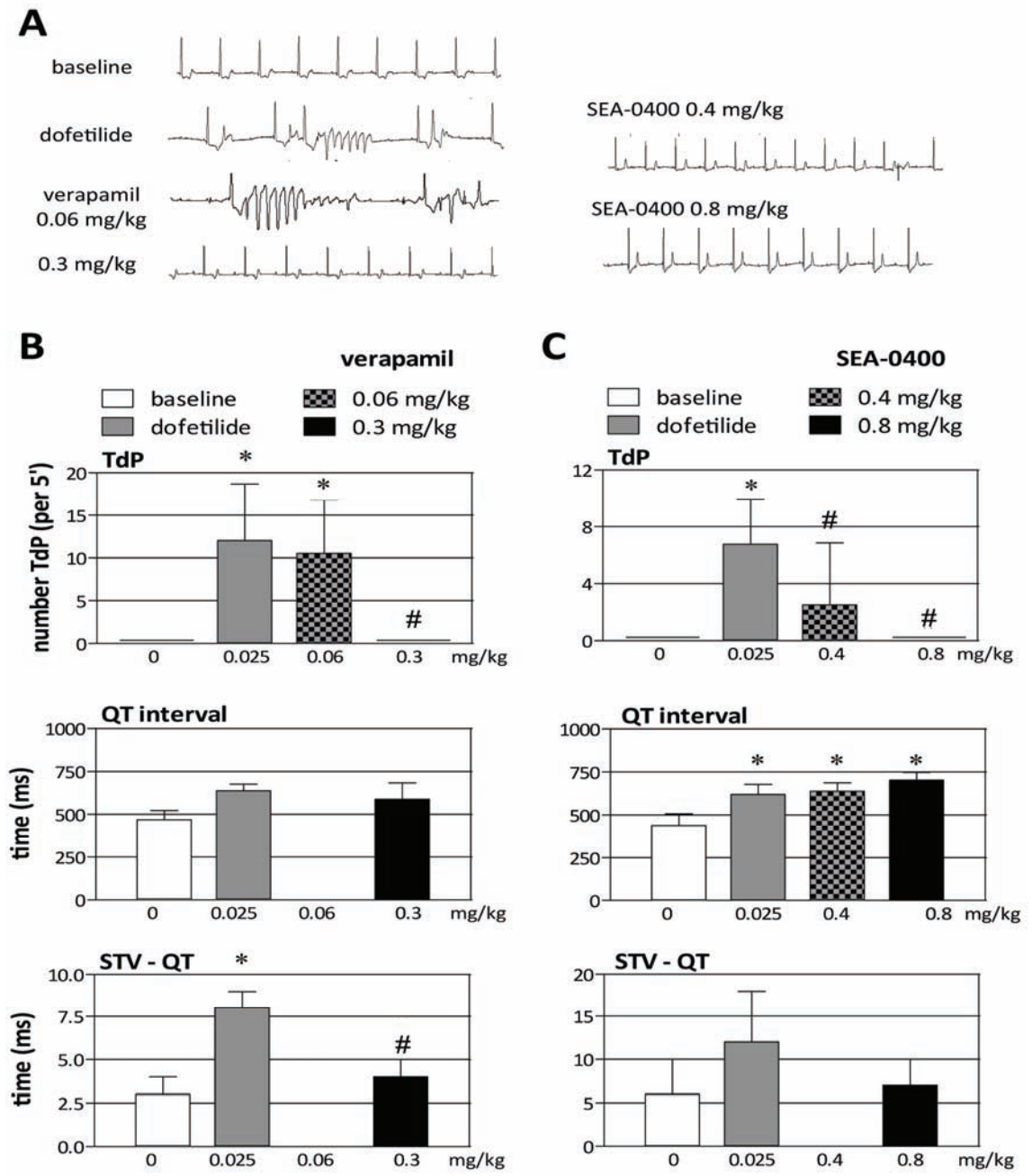

Figure 5. SEA-0400 and verapamil are anti-arrhythmic, but verapamil is only effective at a negative inotropic dose

A, Left, Lead II in a CAVB dog, under baseline, after dofetilide (0.02 mg/kg) showing TdP episodes, and suppression by infusion of 0.3 mg/kg verapamil, but not 0.06 mg/kg. Right, TdP suppression by SEA-0400 (0.4 and 0.8 mg/kg). Note the presence of ectopic beats at the lower dose.

B, Quantification of TdP occurrence, QT-interval (middle) and short-term variability of the QT interval (STV-QT), after treatment with verapamil (N=4).

C, SEA-0400 (N=8 total, half of the dogs received 0.4 mg/kg, the other half 0.8 mg/kg). * = P<0.05 vs. baseline. # = P<0.05 vs. dofetilide. ND = not possible to determine due to arrhythmias.

Discussion

Our data show that SEA-0400, a NCX blocker with additional LTCC inhibition, is an effective anti-arrhythmic against dofetilide-induced EADs and TdPs . It has an advantage over primary block of LTCC with verapamil, another efficient anti-arrhythmic, because of the lack of negative inotropy at equal anti-arrhythmia efficacy.

Clinical need for new drugs in heart failure
With aging of the population and improved post-infarction survival, the number of patients treated for arrhythmias is increasing. Especially in the group with heart failure, there is a growth in number of ICD implants. In recent years, new anti-arrhythmic drugs have been tested to relieve the burden of ICD shock: adjunct therapy. Until now, these trials with azimilide [21] and celivarone [22] have been insufficiently successful for a broad clinical implication. In part this can be attributed to the limitations in dosage of the applied drugs because of adverse effects, based either on pro-arrhythmia or negative inotropism. Therefore there is an unmet need to develop new drugs that are devoid of adverse actions and can be applied in these patient populations.

The CAVB dog model
Induction of chronic, complete AV-block results in ventricular remodeling and encompass molecular and cellular changes at the electrical, contractile (enhanced Ca^{2+} transient) and structural level [18]. In the CAVB dog, the beneficial adaptations that lead to compensated biventricular hypertrophy are counteracted by TdP susceptibility in vivo (e.g. incidence with dofetilide: 75%), and EADs in vitro[3;19]. The model is therefore suited to address the question how an anti-arrhythmic action of SEA-0400 can be combined with maintained LV contractile performance.

Comparison with other anti-arrhythmics in this model
Over the years, numerous anti-arrhythmics have been tested in this model. Considering possible confounding influences as drug dosage and duration of administration, three categories of action can be identified:
1) Ca^{2+} antagonists verapamil and flunarizine are very effective agents that prevent and suppress TdP and EADs.
2) Ranolazine and lidocaine suppress about 60% of the drug induced TdP. Late sodium current inhibition was effective even though its current density was reduced in CAVB dogs as compared to SR. [19]. Despite this, we have also found that subsarcolemmal [Na^+] is probably increased in this model[23]. This is also of importance in identifying the effects of SEA-0400, as higher sodium concentrations promote NCX reverse mode and enhance SEA-0400 NCX block. However, in this model both forward and reverse NCX are increased[24].
3) Drugs like K201 and AVE0118 were not effective at all in controlling these arrhythmias. The superior anti-arrhythmic action of Ca^{2+} antagonists is however accompanied by reduced LV (-26%) and systolic blood pressure (-27%) with flunarizine (2 mg/kg) [25], whereas 0.3 mg/kg verapamil (this study) lowered LVP by 15%. In contrast, the SEA-0400 dosage could be increased to 0.8 mg/kg to have 100% efficacy without compromising LV function.

Mechanisms of anti-arrhythmic activity of SEA-0400

The anti-arrhythmic effect of SEA-0400 was linked to reduced beat-to-beat variability (STV_{QT} or STV_{APD}), while it did not shorten QT-time or APD. The link between variability and TdPs has been well established[3]. Similarly, verapamil also did not shorten the QT-interval, but decreased STV_{QT} and $STV_{LV\ MAPD}$ [3], suggesting that LTCC block is involved in reducing STV and net inward current during the AP plateau. This may directly reduce the likelihood of EADs, related to re-activation of LTCC.

Furthermore, in absence of dofetilide, the inhibition by SEA-0400 is responsible for some AP shortening (Fig.2). In the presence of dofetilide this shortening is no longer apparent, yet STV is reduced. The lack of shortening may be due to the predominant effect of dofetilide, but the shift in balance of currents during the AP plateau, favouring repolarization because of reduced inward current, is presumably still present and thus reduces variability.

Reduced NCX current by itself could also contribute to the observed effects. The role of NCX in EADs is less equivocal than in DADs, but several lines of evidence support its contribution [26] . The reduced variability can also be partly ascribed to Ca^{2+}-dependent activation of NCX during the AP plateau, as intracellular $[Ca^{2+}]_i$ buffering reduces STV after I_K block [13].

SEA-0400 may exert its effects via forward and reverse mode block of NCX as it blocks both modes equally in dog myocytes. This is not unique to the dog, it has previously also been shown in pig[12] and guinea pig[27] .

In summary, both NCX and the LTCC inhibition contribute to the anti-arrhythmic effect of SEA-0400. This mechanism of action complements reported effects of SEA-0400 on DADs through NCX block in isoproterenol-induced arrhythmias[28]. Preliminary data suggest that in the CAVB dog SEA-0400 is also effective on afterdepolarizations related to spontaneous Ca^{2+} release.

Mechanism of preserved LV function: calcium balance

In pig and mouse myocytes, SEA-0400 induced a Ca^{2+} transient [12] increase while others have demonstrated variable effects in dog and rabbit [7;17]. The data underscored that SEA-0400 effects will depend on the prevailing Ca^{2+} fluxes, and balance between LTCC, Ca^{2+} influx and removal by NCX, and SR Ca^{2+} release and re-uptake. This is supported by the observation that in two mouse models of disease (hypertrophy and heart failure), the net effect on Ca^{2+} handling was different from that in healthy hearts[12]. In the hypertrophic remodeling consequent to CAVB, SEA-0400 during 1 Hz pacing did not increase the $[Ca^{2+}]_i$ transient amplitude, though diastolic Ca^{2+} increased slightly.

Using the different kinetics of SEA-0400 for LTCC and NCX, we could demonstrate that the effect of SEA-0400 is the net result of reduced Ca^{2+} release because of LTCC inhibition and gain of Ca^{2+} through inhibition of NCX. Shortening of the AP with the LTCC inhibition also contributes to maintaining Ca^{2+} balance, as a net gain can be observed under voltage clamp (data not shown).

LTCC inhibition is partially inherent to the properties of SEA-0400 (Fig. 1C). However, LTCC inhibition is further enhanced by reduced removal of Ca^{2+} consequent on NCX inhibition. This property may be favourable in protecting against Ca^{2+} overload at higher heart rates.

The cellular data are in line with the preservation of LVP in vivo. However, SEA-0400 did increase diastolic $[Ca^{2+}]_i$ after dofetilide treatment. Data from another study have linked this to diastolic dysfunction [29]. In the present study we did not observe an increase in diastolic pressure. This may be explained by the fact that diastolic function is only partially dependent on relaxation of the myocyte Ca^{2+} transient [30]. Also, vasodilatation leads to lower diastolic pressures, which may be part of SEA-0400 action (see patent: 7183322 Remedy for hypertension). Under control conditions the effects of SEA-0400 on diastolic pressure (table

1) was also negligible, as previously reported [11]. Neither did we see effects on relaxation, quantified as –LV dP/dt (data not shown).

Table 1. Electrophysiological and ventricular pressure changes after SEA-0400 and verapamil

A. SEA-0400	Sinus rhythm		Chronic AVB		Chronic AVB	
dose	0 mg	0.4 mg	0 mg	0.4 mg	0 mg	0.8 mg
HR	95±25	92±27	43±6	42±6	28±15	36±11
QT	264±28	267±31	424±105	411±85	406±48	434±35
QT-STV	1±1	1±1	11±5	11±5	8±7	6±6
LVP_{sys}	96±21	96±20	90±13	90±14	82±28	85±18
LVP_{diast}	3±1	3±1	7±8	5±8	13±12	13±12
N	3	3	3	3	4	4

B. Verapamil	Sinus rhythm		Chronic AVB	
dose	0 mg	0.3 mg	0 mg	0.3 mg
HR	101±9	100±15	42±7	43±5
QT	302±15	304±18	551±59	558±41
QT-STV	0.5±0.2	0.5±0.2	11±6	9±5
LVP_{sys}	70±7	54±4*	97±4	89±2*
LVP_{diast}	4±3	4±2	9±1	7±2
N	3	3	3	3

Table 1: Electrophysiological and LVP changes after SEA-0400 and verapamil infusion
Parameters were determined after 5 min infusion of A) SEA-0400 to a final dose of 0.4 mg/kg (sinus rhythm and CAVB dogs) and 0.8 mg/kg (CAVB dogs only), and B) Verapamil (0.3 mg/kg). HR, heart rate in beats/min; QT and short-term variability of QT-interval, QT-STV, in ms; LVP_{diast} and LVP_{sys}, diastolic and systolic left ventricular pressure, in mm/Hg; N, number of animals. $P<0.05$ vs baseline (0 mg/kg).

Caveats for use of SEA-0400

NCX and LTCC are also modulators of Ca^{2+} balance in cells other than cardiomyocytes, like smooth muscle cells.

Another potential side effect of LTCC blocking drugs is interference with atrioventricular conduction. In CAVB this is difficult to determine, but in three dogs that received SEA-0400 prior to AV-block in sinus rhythm, heart rate (Table 1) and AV conduction (P-R interval went from 110±6 to 108±12) were not affected. Other authors [31] have reported AV-block and cardiac stand still after SEA-0400 infusion, but at a 3.75x higher dose.

The promising results of the present study should not be directly transposed to arrhythmias in other disease models. The CAVB dog is a model for compensated hypertrophy, not heart failure. Others results with SEA-0400 were mixed: positive in an isolated rabbit heart model

of TdP induced with veratridine or sotalol [14], but not with dofetilide [16]. In models of coronary occlusion, arrhythmias were reduced in rat [32], but not in dog [31]. In the guinea pig treated with aconitine, SEA-0400 was not effective [15]. None of these were studies of chronic disease. Given the delicate balance of Ca^{2+} and the different adaptations in e.g. ischemic cardiomyopathy or pressure overload, this will need further study. Also the dose of SEA-0400 has to be taken into account. In our experiments, we went up to 0.8 mg/kg in order to achieve efficacy against TdP without adverse hemodynamic effects. Whether the dose can be further increased is not known and of interest for further studies.

Perspectives

The concept of multiple targets in anti-arrhythmic drugs is not new, and has been used to improve efficacy or minimise side effects. The advantage of SEA-0400 is that it is a *de facto* co-inhibitor, composed into one drug and its targets are known culprits in arrhythmogenesis. In short, the dual block of NCX and LTCC has promise as a safe and effective strategy against repolarization-dependent arrhythmias, with on top of that the important benefit of preserved hemodynamics.

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Disclosures

None.

Reference List

1. Aiba T and Tomaselli GF. Electrical remodeling in the failing heart. Curr. Opin. Cardiol. 2010; 25:29-36.

2. Gupta A, Lawrence AT, Krishnan K, Kavinsky CJ, Trohman RG. Current concepts in the mechanisms and management of drug-induced QT prolongation and torsade de pointes. Am. Heart J. 2007; 153:891-899.

3. Oros A, Houtman MJ, Neco P, Gomez AM, Rajamani S, Oosterhoff P, Attevelt NJ, Beekman JD, van der Heyden MA, Verdonck L, Belardinelli L, Richard S, Antoons G, Vos, MA. Robust anti-arrhythmic efficacy of verapamil and flunarizine against dofetilide-induced TdP arrhythmias is based upon a shared and a different mode of action. Br. J. Pharmacol. 2010; 161:162-175.

4. Shimizu W, Ohe T, Kurita T, Kawade M, Arakaki Y, Aihara N, Kamakura S, Kamiya T, Shimomura, K. Effects of verapamil and propranolol on early afterdepolarizations and ventricular arrhythmias induced by epinephrine in congenital long QT syndrome. J. Am. Coll. Cardiol. 1995; 26:1299-1309.

5. Barry WH, Horowitz JD, Smith TW. Comparison of negative inotropic potency, reversibility, and effects on calcium influx of six calcium channel antagonists in cultured myocardial cells. Br. J. Pharmacol. 1985; 85:51-59.

6. Vigorito C, Giordano A, Ferraro P, Acanfora D, De Capri L, Naddeo C, Rengo F. Hemodynamic effects of magnesium sulfate on the normal human heart. Am. J. Cardiol. 1991; 67:1435-1437.

7. Nagy ZA, Virag L, Toth A, Biliczki P, Acsai K, Banyasz T, Nanasi P, Papp JG, Varro A. Selective inhibition of sodium-calcium exchanger by SEA-0400 decreases early and delayed after depolarization in canine heart. Br. J. Pharmacol. 2004; 143:827-831.

8. Volders PG, Kulcsar A, Vos MA, Sipido KR, Wellens HJ, Lazzara R, Szabo B. Similarities between early and delayed afterdepolarizations induced by isoproterenol in canine ventricular myocytes. Cardiovasc. Res. 1997; 34:348-359.

9. Farkas AS, Acsai K, Nagy N, Toth A, Fulop F, Seprenyi G, Birinyi P, Nanasi PP, Forster T, Csanady M, Papp JG, Varro A, Farkas A. Na(+)/Ca(2+) exchanger inhibition exerts a positive inotropic effect in the rat heart, but fails to influence the contractility of the rabbit heart. Br. J. Pharmacol. 2008; 154:93-104.

10. Tanaka H, Namekata I, Takeda K, Kazama A, Shimizu Y, Moriwaki R, Hirayama W, Sato A, Kawanishi T, Shigenobu K. Unique excitation-contraction characteristics of mouse myocardium as revealed by SEA0400, a specific inhibitor of Na+-Ca2+ exchanger. Naunyn Schmiedebergs Arch. Pharmacol. 2005; 371:526-534.

11. Birinyi P, Toth A, Jona I, Acsai K, Almassy J, Nagy N, Prorok J, Gherasim I, Papp Z, Hertelendi Z, Szentandrassy N, Banyasz T, Fulop F, Papp JG, Varro A, Nanasi PP, Magyar J. The Na+/Ca2+ exchange blocker SEA0400 fails to enhance cytosolic Ca2+ transient and contractility in canine ventricular cardiomyocytes. Cardiovasc. Res. 2008; 78:476-484.

12. Ozdemir S, Bito V, Holemans P, Vinet L, Mercadier JJ, Varro A, Sipido KR. Pharmacological inhibition of na/ca exchange results in increased cellular Ca2+ load attributable to the predominance of forward mode block. Circ. Res. 2008; 102:1398-1405.

13. Johnson DM, Heijman J, Pollard CE, Valentin JP, Crijns HJ, bi-Gerges N, Volders PG. I(Ks) restricts excessive beat-to-beat variability of repolarization during beta-adrenergic receptor stimulation. J. Mol. Cell Cardiol. 2010; 48:122-130.

14. Milberg P, Pott C, Fink M, Frommeyer G, Matsuda T, Baba A, Osada N, Breithardt G, Noble D, Eckardt L. Inhibition of the Na+/Ca2+ exchanger suppresses torsades de pointes in an intact heart model of long QT syndrome-2 and long QT syndrome-3. Heart Rhythm. 2008; 5:1444-1452.

15. Amran MS, Hashimoto K, Homma N. Effects of sodium-calcium exchange inhibitors, KB-R7943 and SEA0400, on aconitine-induced arrhythmias in guinea pigs in vivo, in vitro, and in computer simulation studies. J. Pharmacol. Exp. Ther. 2004; 310:83-89.

16. Farkas AS, Makra P, Csik N, Orosz S, Shattock MJ, Fulop F, Forster T, Csanady M, Papp JG, Varro A, Farkas A. The role of the Na+/Ca2+ exchanger, I(Na) and I(CaL) in the genesis of dofetilide-induced torsades de pointes in isolated, AV-blocked rabbit hearts. Br. J. Pharmacol. 2009; 156:920-932.

17. Milberg P, Pott C, Frommeyer G, Fink M, Ruhe M, Matsuda T, Baba A, Klocke R, Quang TH, Nikol S, Stypmann J, Osada N, Muller FU, Breithardt G, Noble D, Eckardt, L. Acute inhibition of the Na(+)/Ca(2+) exchanger reduces proarrhythmia in an experimental model of chronic heart failure. Heart Rhythm. 2012; 9(4):570-8

18. Oros A, Beekman JD, Vos MA. The canine model with chronic, complete atrio-ventricular block. Pharmacol. Ther. 2007; 119:168-178.

19. Antoons G, Oros A, Beekman JD, Engelen MA, Houtman MJ, Belardinelli L, Stengl M, Vos MA. Late na(+) current inhibition by ranolazine reduces torsades de pointes in the chronic atrioventricular block dog model. J. Am. Coll. Cardiol. 2010; 55:801-809.

20. Antoons G, Volders PG, Stankovicova T, Bito V, Stengl M, Vos MA, Sipido KR. Window Ca2+ current and its modulation by Ca2+ release in hypertrophied cardiac myocytes from dogs with chronic atrioventricular block. J. Physiol. 2007; 579:147-160.

21. Dorian P, Borggrefe M, Al-Khalidi HR, Hohnloser SH, Brum JM, Tatla DS, Brachmann J, Myerburg RJ, Cannom DS, van der, Laan M, Holroyde MJ, Singer I, Pratt CM. Placebo-controlled, randomized clinical trial of azimilide for prevention of ventricular tachyarrhythmias in patients with an implantable cardioverter defibrillator. Circulation. 2004; 110:3646-3654.

22. Gojkovic O, Aliot EM, Capucci A, Connolly SJ, Crijns H, Hohnloser SH, Kulakowski P, Roy D, Radzik D, Singh BN, Kowey PR. Celivarone in patients with an implantable cardioverter-defibrillator: Adjunctive therapy for the reduction of ventricular arrhythmia-triggered implantable cardioverter-defibrillator interventions. Heart Rhythm. 2012; 9:217-224.

23. Verdonck F, Volders PG, Vos MA, Sipido KR. Increased Na+ concentration and altered Na/K pump activity in hypertrophied canine ventricular cells. Cardiovasc. Res. 2003; 57:1035-1043.

24. Sipido KR, Volders PG, de Groot SH, Verdonck F, Van de Werf F, Wellens HJ, Vos MA. Enhanced Ca(2+) release and Na/Ca exchange activity in hypertrophied canine ventricular myocytes: potential link between contractile adaptation and arrhythmogenesis. Circulation. 2000; 102:2137-2144.

25. Vos MA, Gorgels AP, Leunissen JD, van der Nagel T, Halbertsma FJ, Wellens HJ. Further observations to confirm the arrhythmia mechanism-specific effects of flunarizine. J. Cardiovasc. Pharmacol. 1992; 19:682-690.

26. Volders PG, Vos MA, Szabo B, Sipido KR, de Groot SH, Gorgels AP, Wellens HJ, Lazzara R. Progress in the understanding of cardiac early afterdepolarizations and torsades de pointes: time to revise current concepts. Cardiovasc. Res. 2000; 46:376-392.

27. Tanaka H, Nishimaru K, Aikawa T, Hirayama W, Tanaka Y, Shigenobu K. Effect of SEA0400, a novel inhibitor of sodium-calcium exchanger, on myocardial ionic currents. Br. J. Pharmacol. 2002; 135:1096-1100.

28. Fujiwara K, Tanaka H, Mani H, Nakagami T, Takamatsu T. Burst emergence of intracellular Ca2+ waves evokes arrhythmogenic oscillatory depolarization via the Na+-Ca2+ exchanger: simultaneous confocal recording of membrane potential and intracellular Ca2+ in the heart. Circ. Res. 2008; 103:509-518.

29. Schmidt U, Hajjar RJ, Helm PA, Kim CS, Doye AA, Gwathmey JK. Contribution of abnormal sarcoplasmic reticulum ATPase activity to systolic and diastolic dysfunction in human heart failure. J. Mol. Cell Cardiol. 1998; 30:1929-1937.

30. Eberli FR, Stromer H, Ferrell MA, Varma N, Morgan JP, Neubauer S, Apstein CS. Lack of direct role for calcium in ischemic diastolic dysfunction in isolated hearts. Circulation. 2000; 102:2643-2649.

31. Nagasawa Y, Zhu BM, Chen J, Kamiya K, Miyamoto S, Hashimoto K. Effects of SEA0400, a Na+/Ca2+ exchange inhibitor, on ventricular arrhythmias in the in vivo dogs. Eur. J. Pharmacol. 2005; 506:249-255.

32. Takahashi K, Takahashi T, Suzuk, T, Onishi M, Tanaka Y, Hamano-Takahashi A, Ota T, Kameo K, Matsuda T, Baba A. Protective effects of SEA0400, a novel and selective inhibitor of the Na+/Ca2+ exchanger, on myocardial ischemia-reperfusion injuries. Eur. J. Pharmacol. 2003; 458:155-162

Chapter 9:

General discussion

V.J.A. Bourgonje

Abbreviations:
AC3-I: autocamtide-3 related inhibitory peptide
CaMKII: calcium/calmodulin-dependent protein kinase II
CAVB: chronic AV-block
CPVT: catecholaminergic polymorphic ventricular tachycardia
Cx43: connexin 43
DAD(s): delayed afterdepolarization(s)
EAD(s): early afterdepolarization(s)
HDAC: histone deacetylase
LTCC: L-type calcium channel
MEF2: myocyte enhancer factor 2
MHC: myocyte heavy chain
Nav1.5: sodium channel 1.5
NCX: sodium/calcium exchanger
NFAT: nuclear factor of activated T-cells
RyR: ryanodine receptor
SERCA: sarcoplasmic reticulum calcium ATPase
SR: sarcoplasmic reticulum
STV: short term variability
TAC: transverse aortic constriction
TdP: Torsade de Pointes
WT: wildtype

General discussion

The central role of calcium in the heart makes it a tempting, but complex, therapeutic target. Its physiological effects - excitation, contraction, and intracellular signaling - are reflected in the pathophysiological results of intracellular calcium disturbances: arrhythmias, decreased pump function, and detrimental cardiac remodeling.
With this in mind, the research described in this thesis aimed to target calcium-dependent mechanisms in different settings, specifically animal models of arrhythmogenic ventricular remodeling. Two profound problems are 1) that directly targeting calcium via class IV antiarrhythmics is accompanied with negative hemodynamic responses, and 2) that chronic pathological remodeling can result in both pro-arrhythmia and in the progression to heart failure. However, we thought that these problems could be solved through appropriate interference, which we reflected in our general hypothesis:

Targeting calcium is an effective strategy both to acutely inhibit arrhythmias while preserving cardiac function, and to prevent chronic pathological cardiac remodeling

In **chapter 4**, we showed that the onset of TdP in a dog model of long QT was accompanied by CaMKII autophosphorylation. Subsequently, acute CaMKII inhibition effectively inhibited TdP arrhythmias in vivo and EADs in vitro. Surprisingly, this was all observed in a model with compensated hypertrophy, while elevated CaMKII levels and activity are normally associated with decompensated cardiac growth. Another calcium-dependent signaling molecule, calcineurin, appeared neither involved in the arrhythmias, nor in the hypertrophy.
Chapter 5 described the effect of chronic CaMKII inhibition in a mouse model with pressure overload-induced cardiac remodeling. Although the inhibition had no notable consequences for arrhythmogeneity, hypertrophy, or cardiac function, conduction velocity was increased or at least preserved. This could be related to the absence of changes in Cx43 and Nav1.5 expression, but not due to absence of fibrosis.
However, in calcineurin-induced cardiomyopathy, as shown in **chapter 6**, CaMKII inhibition had little effects on conduction velocity related changes, like Cx43, Nav1.5, and fibrosis.
Concerning calcium and arrhythmias, we explained the problems of L-type calcium blockade as an acute treatment in **chapter 7**. Despite their anti-arrhythmic effectiveness, class IV anti-arrhythmics are contra-indicated in situations with decreased cardiac contraction (like in heart failure) due to negative inotropic and hemodynamic effects. This negative side effect, at first sight unavoidable, was, as described in **chapter 8**, proven to be preventable by combining LTCC block with NCX inhibition; decreasing the pro-arrhythmic influx of calcium ions, but nonetheless preserving intracellular calcium concentrations by also inhibiting the efflux of calcium.

In all, it can be concluded that part 1 of the hypothesis is confirmed; calcium block can indeed be anti-arrhythmic while preserving cardiac function. Inhibition of the calcium-dependent protein CaMKII, but not calcineurin, was equally anti-arrhythmic.
However, concerning part 2 of the hypothesis, results are more mixed. Chronic inhibition of signaling via CaMKII had positive effects on conduction velocity after pressure overload, but could not prevent calcineurin-induced pathology. Also, fractional shortening and hypertrophy were not affected. We must therefore conclude that this thesis provides evidence that interfering in calcium-dependent signaling can have positive effects on conduction velocity, but additional beneficial changes, like increased cardiac function, could not be shown. Furthermore, the cause of the cardiac remodeling might influence the effectiveness of inhibition.

1) Calcium and acute arrhythmias

Calcium and CaMKII in long-QT and afterdepolarization dependent arrhythmias

Of all the results shown in this thesis, the acute anti-arrhythmic effect of CaMKII inhibition in the CAVB dog model is probably the least controversial. Both W-7 (in vivo and in vitro) and KN-93 (in vitro) were effective. Although it can be debated whether these pharmaceuticals are completely specific for CaMKII, it is unlikely W-7 and KN-93 have overlapping a-specific effects, considering their different chemical compositions. Furthermore, the effectiveness in long-QT like settings has been confirmed numerous times in the literature[1-3]. In vitro, calcium sparks have been related to CaMKII[4]. All these disease mechanisms can be linked to afterdepolarization-dependent pathologies, as also occur during a prolonged action potential. In all, a robust amount of evidence[5-10] backs up the claim of a beneficial effect of CaMKII block in the treatment against arrhythmias that are depending on long action potentials.
Considering ventricular tachyarrhythmias based on triggered activity (as opposed to re-entry based, which one would expect to see with shorter action potentials), there are two known potential mechanisms for CaMKII to induce these cardiac effects: by directly phosphorylating ion handling proteins and channels[5], and by inducing changes in gene expression via HDAC/MEF2 leading to cardiac remodeling[11, 12]. Since the anti-arrhythmic effect of W-7 in vivo and KN-93 in vitro was within minutes, we can safely discard the second option that would have taken much longer time to induce effects.
The targets that are known to be directly phosphorylated by CaMKII are 1) potassium channels[13], 2) sodium channels[14], and 3) targets related to calcium handling, namely LTCC[5], RyR[4], and phospholamban[4]. Based on currently available evidence in literature, potassium channels are an unlikely candidate, as blocking CaMKII dependent phosphorylation would lead to reduced currents[13], and thus longer action potentials and more risk for arrhythmias. Sodium channels are a potential candidate, as the effect of CaMKII seems to lead to a persistent sodium current, which has been linked to long-QT pathologies[15].
However, the most likely candidate is calcium handling, where CaMKII phosphorylates numerous targets[4, 5]. Recall from **chapter 2** that the mechanism underlying triggered arrhythmias (like ventricular tachyarrhythmias in long-QT situations), is either based on EADs or on DADs. CaMKII can lead to the first by phosphorylating LTCC, giving rise to changed gating properties and potentially window currents, which is by itself depolarizing[16], but that could also be exaggerated by a subsequent inward current via NCX[17].
Secondly, DADs are believed to be caused by calcium sparks; untriggered calcium releases from the SR via RyR[18]. A series of RyRs need to be activated for such a calcium leak to translate in a full blown calcium wave[19], and thus a triggered beat. CaMKII activity increases the open probability of RyR (see **chapter 2**), giving it a fairly straightforward role in both the occurrence of calcium leaks, and the propagation of a small leak to a cell wide calcium wave. Furthermore, CaMKII also phosphorylates phospholamban, thereby reducing its inhibitory effect on SERCA. The net effect will be a higher calcium load of the SR due to increased pump activity of SERCA[19]. This also increases the chance of calcium sparks, as leak is dependent on a high open probability of RyR *and* a high SR calcium load[18]. Next to that, in CPVT, with DAD-dependent arrhythmias, it has been suggested that block of CaMKII is anti-arrhythmic[20].
Thus, there is a clear mechanistic insight in the role of CaMKII both in EADs and DADs, and as such triggered arrhythmias, including the ones observed with long QT intervals.

How CaMKII becomes activated in a long QT setting primarily, is less obvious. After all, the lengthening of the action potential is associated with decreased potassium currents[21], which is unconnected to CaMKII activation. Furthermore, bradycardia, a risk factor in long-QT like settings, is associated with less CaMKII activation[22]. However, a longer action potential can also lead to a longer calcium transient during the beat, which can apparently even overcome bradycardic effects and induce a net activation of CaMKII[22]. Notably, our group has demonstrated previously that a longer action potential alone is not sufficient to induce arrhythmias. An additional necessity is an imbalance in the action potential duration, which we have quantified as the variation in length of subsequent action potentials, averaged over 10-30 beats (STV, short term variability)[23, 24]. It is probably in this phase that (CaMKII induced) calcium disturbances play a role, as inward calcium currents can be translated directly (LTCC)[16] or indirectly (with subsequent NCX activation)[17], into action potential variations. Even further disturbances will then give rise to afterdepolarizations and eventually triggered activity. The evidence suggests a chain of events that is summarized in figure 1.

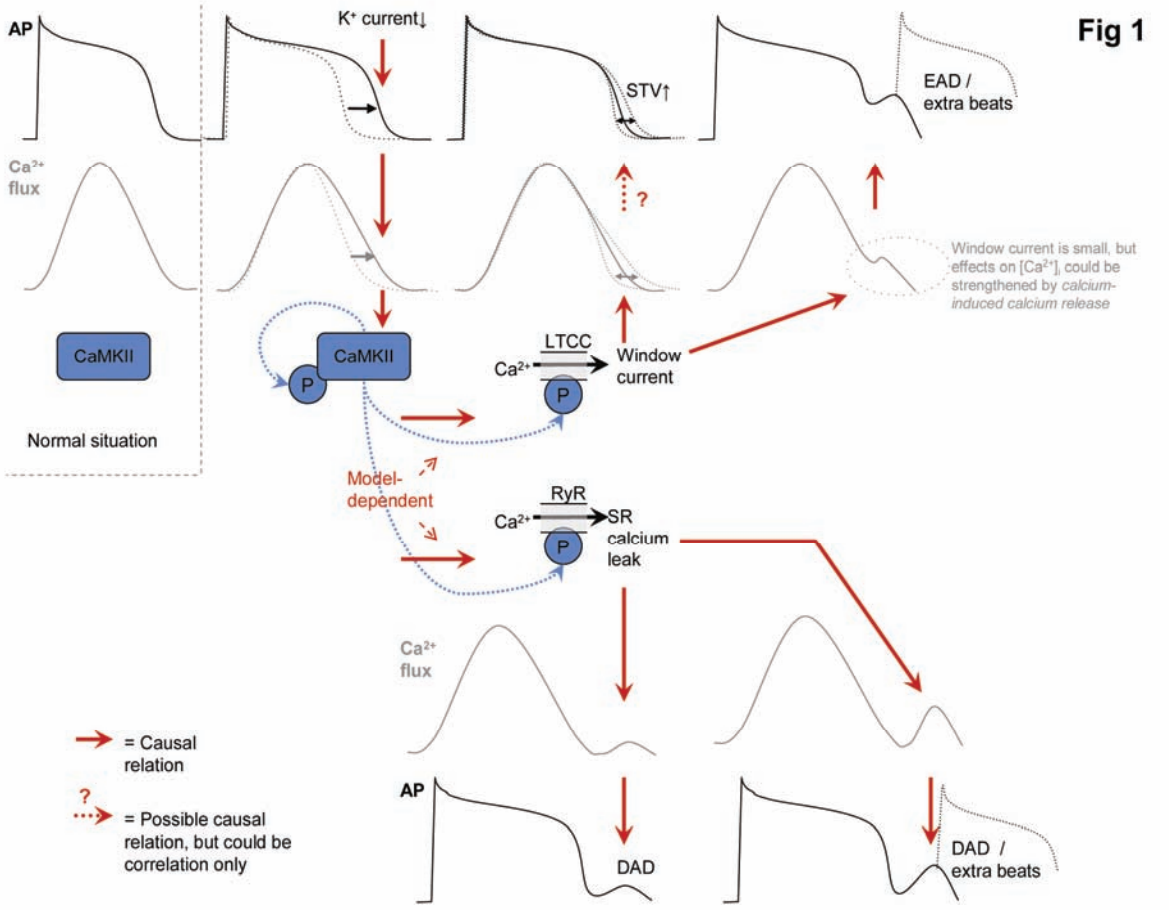


Figure 1: Role of calcium and CaMKII in long-QT dependent arrhythmias
Shown are action potentials (black lines) and cytosolic calcium transients (grey), as well as CaMKII (blue) and two of its targets of phoshporylation (P). dashed lines with an arrow either indicate a lengthening (one-headed arrow) or variation (two-headed arrow) in APD or calcium transient. The red arrows denote the proposed causal relationship in the mechanism of these arrythmias.

Although mechanistically very insightful, a major caveat must be made concerning the therapeutic applicability of acute CaMKII inhibition against long-QT dependent arrhythmias. Leaving aside tissue aspecificity (like affecting CaMKII in other tissues like e.g. the brain)

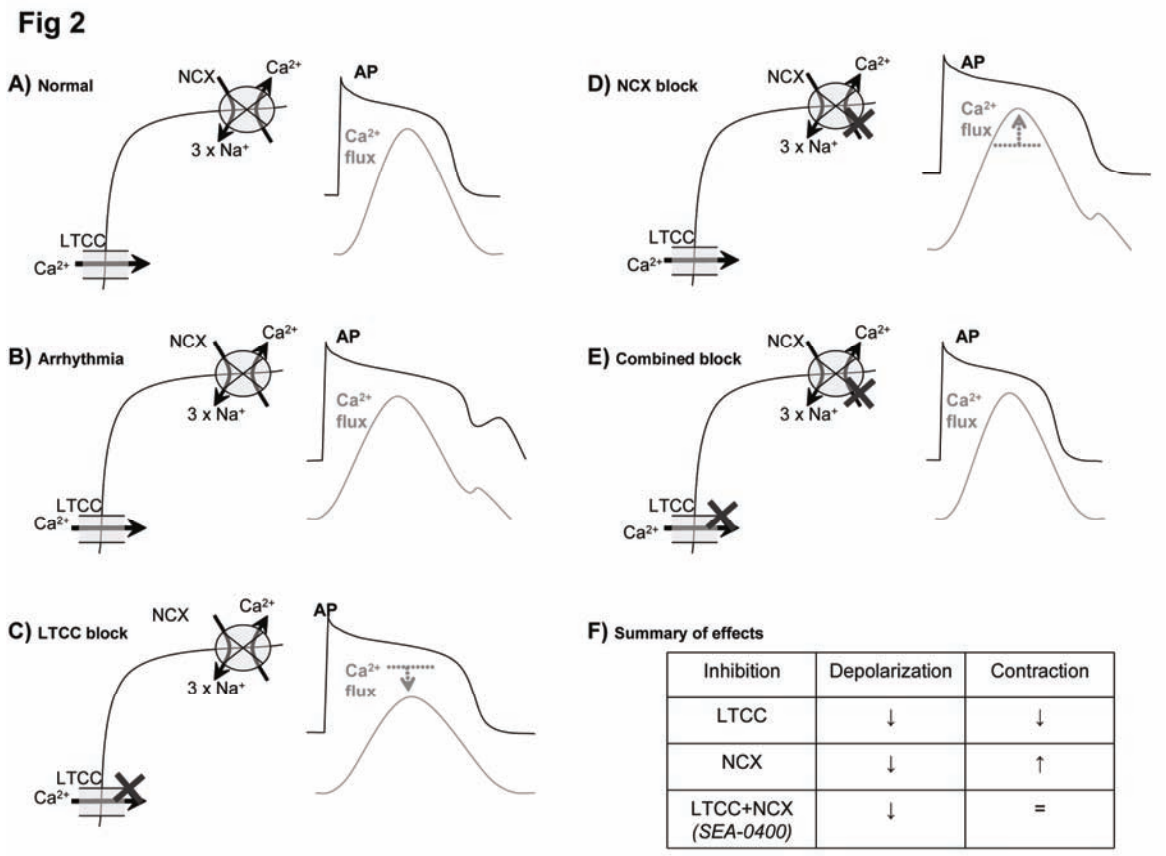
and aspecific effects in the cardiomyocyte itself (a concern for all currently available CaMKII inhibitors), which might be overcome by developing better inhibitors, there is still a structural therapeutic problem. The evidence suggest that CaMKII inhibition is anti-arrhythmic under these circumstances by preventing undesired calcium fluxes via LTCC[25] or RyR[4], or perhaps by diminishing sodium late currents[14], but inhibitors for these currents are already available, like verapamil (LTCC block)[26] or ranolazine (sodium late)[15].
Beneficial effects of targeting CaMKII might be that by targeting indirectly numerous secondary targets at once this strategy is more effective in its anti-arrhythmic activity, or that it reduces side effects, for example by a more balanced change in calcium currents in the cardiomyocyte. The first statement is not true (in the CAVB dog W-7 was partially effective, while verapamil completely suppressed TdPs (see **chapter 7**)), and the second statement is at the moment unproven. CaMKII inhibition as a therapeutic strategy for acutely inhibiting long-QT dependent arrhythmias is, at best, still a long way off.

A better class IV anti-arrhythmic drug?

A more promising strategy to follow for acute anti-arrhythmic treatment is a combined block of LTCC and NCX. Not only appeared this completely anti-arrhythmic in our CAVB dog model, certainly as important, it is inotropically neutral too.
Class IV anti-arrhythmics[27] are very effective, but all come with negative side effects, which can either be blamed on vasodilatation or reduced contractile force of the cardiomyocytes. Combining LTCC block (= anti-arrhythmic) with NCX block (preserves intracellular calcium and thus contraction, might also be anti-arrhythmic) can overcome this.
The drug we tested, SEA-0400, has also been reported to be anti-arrhythmic by others[28], though not by all[29]. However, this last group used a model that is not completely based on a long-QT like mechanism. Verapamil, commonly regarded as truly anti-arrhythmic under these conditions, was in their model equally ineffective. In all, the evidence of an anti-arrhythmic potency of SEA-0400 looks to be robust.
Remarkably, the mechanism underlying its effect is not completely clear. SEA-0400 was developed as a specific NCX blocker[30], but subsequent research has shown it also blocks LTCC by varying degrees (3 to 25%)[31, 32]. This LTCC block seems not only to be direct, but also indirect by increasing intracellular calcium concentrations to which the LTCC open probability is sensitive[32]. LTCC block is anti-arrhythmic, but theoretically, block of the transient inward current of the NCX might be too[33]. At the moment, SEA-0400 cannot make this segregation and both (or a combination of) mechanisms still stand as the potential cause.

A potential limit of SEA-0400 is that it could lead to calcium overload. As it blocks NCX more effectively than LTCC, it inhibits the ouflow of calcium more than the inflow. The direct effect is a slowing of relaxation, which can be a problem by itself, but it could also lead to excessive SR calcium loading, as cytosolic calcium which is not removed by NCX is pumped into the SR via SERCA instead. In this sense, SEA-0400 might not even be the best choice, as its blocking actions are skewed towards the NCX. Paradoxically, the former generation NCX blocker, KB-R7943, might be more suitable because it is less specific[34]. Of an important note though, is that a certain flexibility concerning the ratio LTCC/NCX block might be necessary. In heart failure for example, diastolic calcium is higher and systolic concentrations are lower. With a resulting lower SR calcium load, contraction force is more dependent on LTCC-dependent calcium influx. A number of NCX/LTCC blockers might have to exist together.
Of course, the mechanistic uncertainties considering SEA-0400 are not of the utmost importance in relation to therapeutic feasibility, only whether it works or not, and that it

seems to do. The combination of NCX and LTCC block is thus exactly the opposite of acute CaMKII inhibiton: mechanistically unclear but with lots of therapeutic potential.
The summary of thoughts considering NCX/LTCC inhibition is shown in figure 2.



Inhibition	Depolarization	Contraction
LTCC	↓	↓
NCX	↓	↑
LTCC+NCX *(SEA-0400)*	↓	=

Figure 2: (Combined) block of LTCC and NCX and its effects on EADs and calcium transients. A) denotes the normal situation, B) with arrhythmia, C) LTCC block, D) NCX block, E) combined block, and F) shows a summary of effects n the table.

2) Calcium and cardiac remodeling

CaMKII & calcineurin in hypertrophy

Next to excitation and contraction, calcium is, as explained (see **chapter 3**), involved in intracellular signaling. In that respect, this thesis focused mainly on the downstream effects of two calcium-dependent signaling molecules, calcineurin and CaMKII.
While the acute effects of CaMKII in arrhythmias are due to direct phosphorylation of ion channels and thus ion current changes, the chronic effects evoked by CaMKII are also believed to induce hypertrophy and heart failure.
Firstly, CaMKII expression and activation are reported to be increased in human heart failure[35]. Secondly, overexpression of CaMKII in a mouse model induces dilated cardiomyopathy[36]. Thirdly, CaMKII knock out possibly rescues hypertrophy after a challenge (HW/BW rescued in Backs et al.[37] and Lu et al.[38], but not in Ling et al[39]), as well as dilatation and fractional shortening[37-39].

A general distillation from literature implies a mechanism where CaMKII is activated directly via increased calcium levels, and thus indirectly via mechanisms that increase calcium in the first place, like chronic adrenergic stimulation. A second option is direct oxidation of CaMKII by reactive oxygen species, which also activates CaMKII (like autophosphorylation), albeit slightly less effective. Calcium is still needed to induce the first activation though, as the oxidation event happens with calcium/calmoduling bound; only thereafter can oxidized CaMKII function calcium-independently. As an example underlying this second mode of activation, it has been suggested that chronic elevated angiotensin II levels can be responsible for increased radical formation.[40]
Once chronically active, nuclear CaMKII can phosphorylate HDAC4, inducing translocation out of the nucleus[41]. Cytosolic CaMKII can then subsequently keep these proteins confined to the cytosol. Nuclear HDAC4 normally inhibits MEF2-depent gene transcription, and with HDAC4 being removed MEF2 sensitive genes become activated leading to a specific expression pattern which includes a shift to β-MHC and expression of atrial natriuretic peptide, amongst others. This pattern is associated with, and has been implicated as a cause for, dilated cardiomyopathy[11].
Our results from the CAVB dog are not completely in agreement. Although the CAVB dog displays chronic autophosphorylation of CaMKII (and thus activation), the observed hypertrophy is compensated, with preserved cardiac output[42]. In agreement with this, adrenergic and ANP levels are only transiently elevated[42], which implies the heart only experiences a temporal state of stress. In accordance with this, we have shown that despite higher CaMKII activity, HDAC4 was not more phosphorylated. The pathway towards MEF2-dependent gene expression was thus arrested, and therefore probably the deterioration into heart failure avoided.

A striking difference with HF in the CAVB dog, and a potential explanation for the lack of HDAC phosphorylation, is that the total protein expression levels of CaMKII are not increased. Perhaps in the CAVB dog, CaMKII activity remains within physiological range, thereby increasing the calcium transient (higher maximum cytosolic levels, but also fast removal and thus low diastolic levels), which is beneficial for systolic contraction and diastolic relaxation (although it is pro-arrhythmic[17]), while in decompensated situations the increase of CaMKII levels and activity can give rise to strongly increased signals that exceed threshold since they are far beyond homeostatic levels.
Obviously, in the compensated CAVB dog, the CaMKII activity is compartmentalized, with autophosphorylation, and phosphorylation of phospholamban, but not of HDAC4. The mechanism behind this is unknown at the moment, and it could be speculated to be due to numerous causes, like specific phosphatase activity or reduced nuclear CaMKII activity (perhaps by lower nuclear calcium levels). The latter suggestion, differences between cytosolic and nuclear calcium levels and their regulation, is also an aspect with more questions than answers.
The results we observed in the mice with aortic constriction and chronic CaMKII inhibition (**chapter 5**) were surprising and not anticipated: inhibition did not prevent hypertrophy nor reduce fractional shortening. Although technically not dilated cardiomyopathy (wall thickness also increased), these mice clearly had a deteriorated heart function, likewise in mice without inhibition of CaMKII. As all available literature notices increased CaMKII activation and expression after TAC[38, 39, 43-45] and CaMKII knock-out has been reported to rescue cardiac function[37-39], there is a discrepancy that needs an explanation. (Table 1 and Table 2)
One option is that CaMKII knock-out is different than inhibition via AC3-I: knock-out might also disturb macromolecular complexes in which CaMKII normally functions, while AC3-I would probably leave these intact. However, a more probable explanation is time: we checked

for cardiac function at 16 weeks post TAC-surgery, while other groups analyzed much earlier, varying from 7 to 56 days. It is reasonable to suggest that chronic CaMKII inhibition does not prevent cardiac deterioration, but in stead slows the onset of problems. That inhibiting just one signaling protein could prevent heart failure completely might be an unrealistically optimistic viewpoint, especially since there are more signaling proteins involved in deterioration to heart failure. Chronic activation of the calcium sensitive phosphatase calcineurin, for example, has been shown to lead to comparable effects: overexpression will give rise to dilated cardiomyopathy[46] and in heart failure its expression is increased[47]. The pathway is also quite well established; activation of calcineurin by calcium/calmodulin leads to dephosphorylation of NFAT, a transcription factor that subsequently translocates to the nucleus where it induces a gene transcription program, including a shift to β-MHC[48].
In our mouse model[49] (**chapter 6**) overexpression of calcineurin indeed leads to heart failure, even with CaMKII inhibition present. Like CaMKII, the calcineurin pathway leading to gene expression changes is not believed to be involved in compensated hypertrophy, as inhibition had no effect on exercise induced hypertrophy in mice[50]. Unlike CaMKII, calcineurin has probably no acute involvement in ion handling. Acute inhibition was also not anti-arrhythmic in our CAVB dog model.
It has been suggested that these signaling pathways become active before structural changes occur in the heart, but that once activated, the heart is committed to a decompensated state (see **chapter 3, figure 1 and 2).** Since the decompensated heart has severe calcium disturbances, like increased diastolic $[Ca^{2+}]_{cytosol}$ and SR calcium leak, as well as elevated adrenergic and angiotensin levels, it is understandable that in the terminal state both calcineurin and CaMKII pathways are active, unconnected to the original initiating event of the pathology. In this way the pathways are indirectly linked. A direct connection however, is unlikely: calcineurin does not dephosphorylate CaMKII, and CaMKII will probably not phosporylate calcineurin. Importantly, heart failure is not only associated with increased activity of calcineurin and CaMKII, but with increased expression as well, implying a spiral with increased activity leading to increased expression and thus even more activity.
Our ideas suggesting calcineurin and CaMKII leading to (de)compensated hypertrophy are summarized in figure 3. Detailed breakdown of the pathways can also be found in **chapter 3, figure 2**.

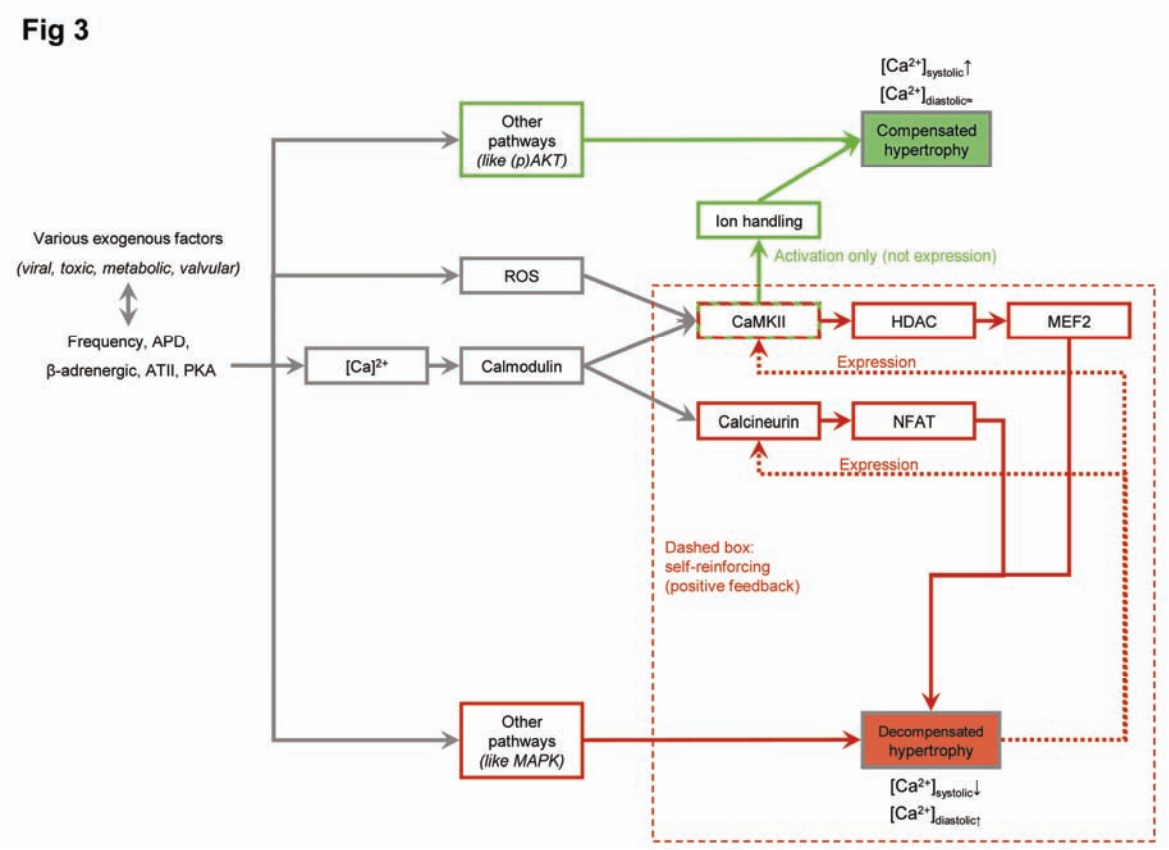


Figure 3: Differences in calcium signaling with regard to compensated (green) and decompensated (red) hypertrophy. Arrows show the direction of signal. Dotted lines indicate a feedback mechanism via increased expression of calcineurin and CaMKII.

Reference:	TAC	TAC induced CaMKII activation	HW/BW or TL	Dilatation	Fractional shortening	Arrhythmias	Conduction velocity
Backs et al., PNAS, 2009	21 days	-	↑ (+53%)	-	-	-	-
Colomer et al., Mol Endocrinol., 2003	7 days	↑ (+33% expression, +50% activity)	↑ (+82%)	-	-	-	-
Hsu et al., Cardiovasc Res., 2009	21 days	↑ (+100% autophosphorylation)	↑ (+100%)	↑ (+25% End-diastolic dimension	↓ -30%	-	-
Ling et al., JCI, 2009	14 days	↑ (+50% expression, +70% autophosphorylation)	↑ (+70%)	↑ (+15% LVIDd)	↓ -7%	-	-
Van Oort, Circulation, 2010	56 days	-	-	-	-	↑ (75% incidence PES-induced NSVT)	-
Lu et al. PLoS One, 2011	56 days	↑ (+200% autophosphorylation)	↑ (+59%)	↑ (+60% chamber diameter)	↓ -40%	-	-
Toischer et al. Circulation, 2010	56 days	↑ (+100% autophosphorylation)	↑ (+22% after 7 days)	↑ (+13% LVEDD)	↓ -20%	-	-
Our group (Jansen et al.) – (Boulaksil et al.)	112 days	-	↑ (+23 - 39%))	↑(+12 - 25%)	↓ (-17 - 23%)	↑ (17-43% pacing-induces SVT)	↓ (RV longitudinal: -23 – 24%)

Table 1: Summary of described changes (in literature) after TAC in mice

Reference:	TAC	CaMKII inhibition	HW/BW or TL	Dilatation	Fractional shortening	Arrhythmias	Conduction velocity
Backs et al., PNAS, 2009	21 days	Knock out	↓	-	-	-	-
Ling et al., JCI, 2009	14 days	Knock out	=	↓	↑	Less Calcium sparks	-
Lu et al. PLoS One, 2011	56 days	DY-9836	↓	-	↑	-	-
Bourgonje et al.	112 days	AC3-I	=	=	=	=	↑

Table 2: Summary of the effects of CaMKII inhibition in TAC mice

CaMKII, calcineurin, and conduction velocity

As far as we know, a link between CaMKII and conduction velocity has never before been reported. In **chapter 5**, we show that chronic CaMKII inhibition rescues conduction velocity after TAC (and perhaps even a slight effect at baseline conduction). This might have to do with preservation of Nav1.5 and Cx43 expression, although the exact mechanism eludes us at the moment. Rescue of fibrosis levels is no participating factor, as this was still increased after TAC, regardless of the CaMKII inhibition. It would be interesting to explore if the underlying factor is a stabilization of the intercalated disc, as is for example proposed in the recent thesis of Dr. Noorman. (See for example Noorman et al., Heart Rhythm, 2012.[51])
Complicating is that the diminished expression of Cx43 and Nav1.5 as observed in mice with calcineurin overexpression (**chapter 6**) could not be prevented by chronic CaMKII inhibition. This could either be because of a different mechanism in this model; that in TAC Nav1.5 and Cx43 expression is CaMKII dependent, while in the calcineurin mouse it is not. Or, possibly, that the calcineurin expression is so extremely high, that CaMKII inhibition will have only a negligible effect in this much more severe model of heart failure. Khoo et al.[52] reported CaMKII inhibition to be anti-arrhythmic in this model, which contradicts with our results. However, their anti-arrhythmic effect was modest and only under certain circumstances (a partial reduction in pacing induced arrhythmias, no effect on spontaneous arrhythmias), implying that the CaMKII anti-arrhythmic effect is at best mild.
Concerning calcineurin and conduction, the direct link is also not clear. Overexpressing calcineurin will lead to disturbed Cx43 and Nav1.5 expression, as well as fibrosis, and this could very well lead to conduction problems, but exactly how calcineurin interacts with Cx43, Nav1.5, and fibrosis is not yet determined. It could even be completely indirect, where calcineurin induces severe cardiomyopathy, which is accompanied by activation of numerous pathways (like CaMKII) linked to heart failure. One of these pathways might then be the cause for the changes in Cx43, Nav1.5, or the induction of fibrosis. The exact mechanism linking calcineurin, CaMKII, and conduction velocity, is still not fully elucidated, but the connection is intriguing and novel.

Conduction velocity and arrhythmias in the pressure overloaded mouse model

It was highly surprising that CaMKII inhibition improved conduction velocity in the TAC model. Equally, if not more, surprising was that this rescue of conduction was not accompanied by reduced arrhythmogeneity. In previous work, our group has tried to unravel the mechanism behind the arrhythmias in TAC mice. In general, the main mechanism was believed to be re-entry. This was based on the observations of severe conduction slowing and sometimes the capture of re-entry like conduction patterns with epicardial electrical mapping[53].
As cause for the conduction slowing, disturbances in Cx43[54] and Nav1.5[55] expression have been suggested, as well as an increase in fibrosis[56]. Later on, this was refined to heterogeneity of expression[57]. The idea was that conduction slowing by itself, as long as it was homogenous, was not dangerous. Only disturbed patterns of conduction lead to a real risk of arrhythmias. Furthermore, the combined message of a number of articles[57-61] seems to suggest that for conduction to slow a change in just one of the involved factors (Cx43, Nav1.5, fibrosis) is not sufficient, but that multiple have to deteriorate in tandem, implying a conduction reserve. It is not straightforward to mirror our results to this framework. CaMKII rescues conduction velocity, and there are no Cx43 and Nav1.5 abnormalities. Fibrosis was still increased after TAC, but this fits with the idea that only one deteriorated factor is not sufficient for significant slowing. Unpublished data from our department reveal that in two other models of

CaMKII inhibition, Cx43 expression and conduction are rescued too (Takanari et al., abstract submitted to Heart Rhythm 2013, Denver, USA).
However, despite rescued conduction, arrhythmias were still there. This is a discrepancy. If arrhythmias were re-entry based, rescue of the conduction velocity would have a significant anti-arrhythmic effect. Also, of all the arrhythmic beats in the AC3-I mice, only one showed a re-entry like pattern while the others had rapid conduction. Observed arrhythmias were also polymorphic, disproving an involvement of stable re-entry patterns (but not unstable, shifting re-entry). This would suggest that the arrhythmias observed in TAC mice with CaMKII inhibition are based on triggered activity.
How to reconcile this with the importance of conduction velocity in WT TAC mice? To speculate, it could be that CaMKII inhibition would give rise to arrhythmias not seen in WT TAC mice, but this seems unlikely. CaMKII inhibition has never been observed to lead to arrhythmias (although this does not mean that it is always anti-arrhythmic either).
One way to reconcile this is to suggest a mechanism where the arrhythmias are induced via triggered activity, and re-entry mechanism might then be involved in perpetuation. After all, the observation of reduced conduction velocity and re-entry characteristics in WT mice still stands. Before, it would be impossible to discriminate between this suggestion and a re-entry only mechanism, as conduction velocity has never been rescued in the TAC mouse previously. The correlation between arrhythmias and conduction velocity is certainly there in WT, but a causal link has now become less certain. However, since there are signs of re-entry in WT TAC, it probably does play a role, albeit a secondary one. This idea is summarized in figure 4.

Fig 4

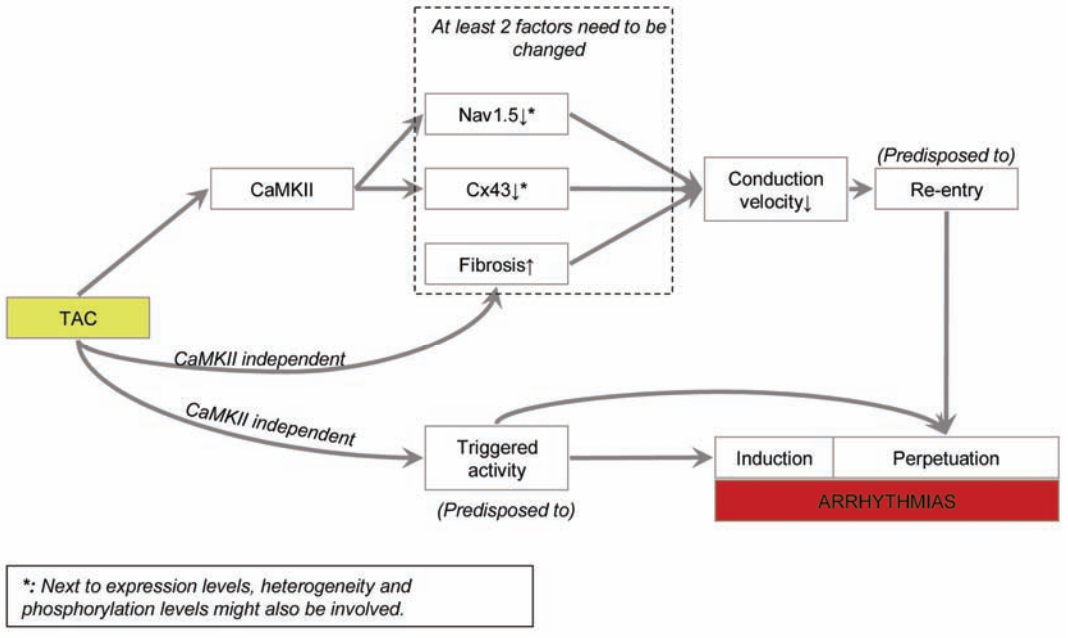


Figure 4: CaMKII dependent and independent effects in mice with pressure overload (TAC)

Summary & Conclusion

Targeting calcium in the heart is tempting due to its multiple roles, but therefore also a complicated target. Our results reflect this. Considering the role of calcium in afterdepolarizations it was quite understandable that targeting calcium handling via CaMKII would be anti-arrhythmic in long-QT settings, where afterdepolarizations (based on calcium currents) are part of the arrhythmic mechanism. However, so far there is no reason to believe this is more beneficial than targeting calcium directly via class IV anti-arrhythmics.
In that regard, SEA-0400 shows much more promise. It is just as effective as available calcium blockers, but without the maladaptive negative inotropy. By smart manipulation of the calcium flux, it might be possible to affect excitation without influencing contraction too much after all.
The complexity of calcium-dependent signaling was especially faced when looking at the effects of chronic calcineurin and CaMKII activation and inhibition. While literature suggests that CaMKII inhibition protects against heart failure, we have to conclude that, based on our obtained results, this is an oversimplification. It is probably more appropriate to state that it is partially effective under some circumstances. Similarly, anti-arrhythmic effects of CaMKII inhibition are situation dependent as well: yes in long-QT, no in pressure-overload induced changes. Concerning the effects of CaMKII inhibition on conduction velocity the same caveat stands: yes in pressure-overload induced hypertrophy, no in calcineurin-overexpression induced hypertrophy.
Thus, concerning acute pro-arrhythmic calcium currents, we can conclude that they are complex indeed, but that our current understanding is deep enough to be close to effective interference. Concerning chronic interference in calcium-dependent signaling though, however promising, much more experimental support is needed to reliable predict the effects of targeting these pathways. Continuous refinement should make it possible to decipher the chronic effects of calcium (inhibition) in the future. Then, targeting calcium will not only be tempting and complex, but also effective and understood.

Reference List

(1) Qi X, Yeh YH, Chartier D et al. The calcium/calmodulin/kinase system and arrhythmogenic afterdepolarizations in bradycardia-related acquired long-QT syndrome. *Circ Arrhythm Electrophysiol* 2009 June;2(3):295-304.

(2) Tsuji Y, Opthof T, Yasui K et al. Ionic mechanisms of acquired QT prolongation and torsades de pointes in rabbits with chronic complete atrioventricular block. *Circulation* 2002 October 8;106(15):2012-8.

(3) Tsuji Y, Hojo M, Voigt N et al. Ca(2+)-related signaling and protein phosphorylation abnormalities play central roles in a new experimental model of electrical storm. *Circulation* 2011 May 24;123(20):2192-203.

(4) Maier LS, Zhang T, Chen L, DeSantiago J, Brown JH, Bers DM. Transgenic CaMKIIdeltaC overexpression uniquely alters cardiac myocyte Ca2+ handling: reduced SR Ca2+ load and activated SR Ca2+ release. *Circ Res* 2003 May 2;92(8):904-11.

(5) Anderson ME, Braun AP, Wu Y et al. KN-93, an inhibitor of multifunctional Ca++/calmodulin-dependent protein kinase, decreases early afterdepolarizations in rabbit heart. *J Pharmacol Exp Ther* 1998 December;287(3):996-1006.

(6) Gbadebo TD, Trimble RW, Khoo MS, Temple J, Roden DM, Anderson ME. Calmodulin inhibitor W-7 unmasks a novel electrocardiographic parameter that predicts initiation of torsade de pointes. *Circulation* 2002 February 12;105(6):770-4.

(7) Kirchhof P, Fabritz L, Kilic A, Begrow F, Breithardt G, Kuhn M. Ventricular arrhythmias, increased cardiac calmodulin kinase II expression, and altered repolarization kinetics in ANP receptor deficient mice. *J Mol Cell Cardiol* 2004 May;36(5):691-700.

(8) Lu HR, Vlaminckx E, Van de Water A, Gallacher DJ. Calmodulin antagonist W-7 prevents sparfloxacin-induced early afterdepolarizations (EADs) in isolated rabbit purkinje fibers: importance of beat-to-beat instability of the repolarization. *J Cardiovasc Electrophysiol* 2006 April;17(4):415-22.

(9) Mazur A, Roden DM, Anderson ME. Systemic administration of calmodulin antagonist W-7 or protein kinase A inhibitor H-8 prevents torsade de pointes in rabbits. *Circulation* 1999 December 14;100(24):2437-42.

(10) Wu Y, Temple J, Zhang R et al. Calmodulin kinase II and arrhythmias in a mouse model of cardiac hypertrophy. *Circulation* 2002 September 3;106(10):1288-93.

(11) Zhang T, Kohlhaas M, Backs J et al. CaMKIIdelta isoforms differentially affect calcium handling but similarly regulate HDAC/MEF2 transcriptional responses. *J Biol Chem* 2007 November 30;282(48):35078-87.

(12) Ling H, Zhang T, Pereira L et al. Requirement for Ca2+/calmodulin-dependent kinase II in the transition from pressure overload-induced cardiac hypertrophy to heart failure in mice. *J Clin Invest* 2009 May;119(5):1230-40.

(13) Wagner S, Hacker E, Grandi E et al. Ca/calmodulin kinase II differentially modulates potassium currents. *Circ Arrhythm Electrophysiol* 2009 June;2(3):285-94.

(14) Wagner S, Dybkova N, Rasenack EC et al. Ca2+/calmodulin-dependent protein kinase II regulates cardiac Na+ channels. *J Clin Invest* 2006 December;116(12):3127-38.

(15) Antoons G, Oros A, Beekman JD et al. Late na(+) current inhibition by ranolazine reduces torsades de pointes in the chronic atrioventricular block dog model. *J Am Coll Cardiol* 2010 February 23;55(8):801-9.

(16) Antoons G, Volders PG, Stankovicova T et al. Window Ca2+ current and its modulation by Ca2+ release in hypertrophied cardiac myocytes from dogs with chronic atrioventricular block. *J Physiol* 2007 February 15;579(Pt 1):147-60.

(17) Sipido KR, Volders PG, de Groot SH et al. Enhanced Ca(2+) release and Na/Ca exchange activity in hypertrophied canine ventricular myocytes: potential link between contractile adaptation and arrhythmogenesis. *Circulation* 2000 October 24;102(17):2137-44.

(18) Antoons G, Oros A, Bito V, Sipido KR, Vos MA. Cellular basis for triggered ventricular arrhythmias that occur in the setting of compensated hypertrophy and heart failure: considerations for diagnosis and treatment. *J Electrocardiol* 2007 November;40(6 Suppl):S8-14.

(19) Bers DM. Cardiac excitation-contraction coupling. *Nature* 2002 January 10;415(6868):198-205.

(20) Liu N, Ruan Y, Denegri M et al. Calmodulin kinase II inhibition prevents arrhythmias in RyR2(R4496C+/-) mice with catecholaminergic polymorphic ventricular tachycardia. *J Mol Cell Cardiol* 2011 January;50(1):214-22.

(21) Volders PG, Sipido KR, Vos MA et al. Downregulation of delayed rectifier K(+) currents in dogs with chronic complete atrioventricular block and acquired torsades de pointes. *Circulation* 1999 December 14;100(24):2455-61.

(22) Livshitz LM, Rudy Y. Regulation of Ca2+ and electrical alternans in cardiac myocytes: role of CAMKII and repolarizing currents. *Am J Physiol Heart Circ Physiol* 2007 June;292(6):H2854-H2866.

(23) Oosterhoff P, Oros A, Vos MA. Beat-to-beat variability of repolarization: a new parameter to determine arrhythmic risk of an individual or identify proarrhythmic drugs. *Anadolu Kardiyol Derg* 2007 July;7 Suppl 1:73-8.

(24) Thomsen MB, Verduyn SC, Stengl M et al. Increased short-term variability of repolarization predicts d-sotalol-induced torsades de pointes in dogs. *Circulation* 2004 October 19;110(16):2453-9.

(25) Dzhura I, Wu Y, Colbran RJ, Balser JR, Anderson ME. Calmodulin kinase determines calcium-dependent facilitation of L-type calcium channels. *Nat Cell Biol* 2000 March;2(3):173-7.

(26) Oros A, Houtman MJ, Neco P et al. Robust anti-arrhythmic efficacy of verapamil and flunarizine against dofetilide-induced TdP arrhythmias is based upon a shared and a different mode of action. *Br J Pharmacol* 2010 September;161(1):162-75.

(27) Zipes DP, Camm AJ, Borggrefe M et al. ACC/AHA/ESC 2006 Guidelines for Management of Patients With Ventricular Arrhythmias and the Prevention of Sudden Cardiac Death: a report of the American College of Cardiology/American Heart Association Task Force and the European Society of Cardiology Committee for Practice Guidelines (writing committee to develop Guidelines for Management of Patients With Ventricular Arrhythmias and the Prevention of Sudden Cardiac Death): developed in collaboration with the European Heart Rhythm Association and the Heart Rhythm Society. *Circulation* 2006 September 5;114(10):e385-e484.

(28) Milberg P, Pott C, Fink M et al. Inhibition of the Na+/Ca2+ exchanger suppresses torsades de pointes in an intact heart model of long QT syndrome-2 and long QT syndrome-3. *Heart Rhythm* 2008 October;5(10):1444-52.

(29) Amran MS, Hashimoto K, Homma N. Effects of sodium-calcium exchange inhibitors, KB-R7943 and SEA0400, on aconitine-induced arrhythmias in guinea pigs in vivo, in vitro, and in computer simulation studies. *J Pharmacol Exp Ther* 2004 July;310(1):83-9.

(30) Matsuda T, Arakawa N, Takuma K et al. SEA0400, a novel and selective inhibitor of the Na+-Ca2+ exchanger, attenuates reperfusion injury in the in vitro and in vivo cerebral ischemic models. *J Pharmacol Exp Ther* 2001 July;298(1):249-56.

(31) Nagy ZA, Virag L, Toth A et al. Selective inhibition of sodium-calcium exchanger by SEA-0400 decreases early and delayed after depolarization in canine heart. *Br J Pharmacol* 2004 December;143(7):827-31.

(32) Ozdemir S, Bito V, Holemans P et al. Pharmacological inhibition of na/ca exchange results in increased cellular Ca2+ load attributable to the predominance of forward mode block. *Circ Res* 2008 June 6;102(11):1398-405.

(33) Antoons G, Willems R, Sipido KR. Alternative strategies in arrhythmia therapy: evaluation of Na/Ca exchange as an anti-arrhythmic target. *Pharmacol Ther* 2012 April;134(1):26-42.

(34) Tanaka H, Nishimaru K, Aikawa T, Hirayama W, Tanaka Y, Shigenobu K. Effect of SEA0400, a novel inhibitor of sodium-calcium exchanger, on myocardial ionic currents. *Br J Pharmacol* 2002 March;135(5):1096-100.

(35) Hoch B, Meyer R, Hetzer R, Krause EG, Karczewski P. Identification and expression of delta-isoforms of the multifunctional Ca2+/calmodulin-dependent protein kinase in failing and nonfailing human myocardium. *Circ Res* 1999 April 2;84(6):713-21.

(36) Zhang T, Johnson EN, Gu Y et al. The cardiac-specific nuclear delta(B) isoform of Ca2+/calmodulin-dependent protein kinase II induces hypertrophy and dilated cardiomyopathy associated with increased protein phosphatase 2A activity. *J Biol Chem* 2002 January 11;277(2):1261-7.

(37) Backs J, Backs T, Neef S et al. The delta isoform of CaM kinase II is required for pathological cardiac hypertrophy and remodeling after pressure overload. *Proc Natl Acad Sci U S A* 2009 February 17;106(7):2342-7.

(38) Lu YM, Huang J, Shioda N et al. CaMKIIdeltaB mediates aberrant NCX1 expression and the imbalance of NCX1/SERCA in transverse aortic constriction-induced failing heart. *PLoS One* 2011;6(9):e24724.

(39) Ling H, Zhang T, Pereira L et al. Requirement for Ca2+/calmodulin-dependent kinase II in the transition from pressure overload-induced cardiac hypertrophy to heart failure in mice. *J Clin Invest* 2009 May;119(5):1230-40.

(40) Erickson JR, Joiner ML, Guan X et al. A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. *Cell* 2008 May 2;133(3):462-74.

(41) Little GH, Bai Y, Williams T, Poizat C. Nuclear calcium/calmodulin-dependent protein kinase IIdelta preferentially transmits signals to histone deacetylase 4 in cardiac cells. *J Biol Chem* 2007 March 9;282(10):7219-31.

(42) Vos MA, de Groot SH, Verduyn SC et al. Enhanced susceptibility for acquired torsade de pointes arrhythmias in the dog with chronic, complete AV block is related to cardiac hypertrophy and electrical remodeling. *Circulation* 1998 September 15;98(11):1125-35.

(43) Colomer JM, Mao L, Rockman HA, Means AR. Pressure overload selectively up-regulates Ca2+/calmodulin-dependent protein kinase II in vivo. *Mol Endocrinol* 2003 February;17(2):183-92.

(44) Hsu S, Nagayama T, Koitabashi N et al. Phosphodiesterase 5 inhibition blocks pressure overload-induced cardiac hypertrophy independent of the calcineurin pathway. *Cardiovasc Res* 2009 February 1;81(2):301-9.

(45) Toischer K, Rokita AG, Unsold B et al. Differential cardiac remodeling in preload versus afterload. *Circulation* 2010 September 7;122(10):993-1003.

(46) Molkentin JD, Lu JR, Antos CL et al. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* 1998 April 17;93(2):215-28.

(47) Lim HW, Molkentin JD. Calcineurin and human heart failure. *Nat Med* 1999 March;5(3):246-7.

(48) van Rooij E, Doevendans PA, de Theije CC, Babiker FA, Molkentin JD, De Windt LJ. Requirement of nuclear factor of activated T-cells in calcineurin-mediated cardiomyocyte hypertrophy. *J Biol Chem* 2002 December 13;277(50):48617-26.

(49) Bierhuizen MF, Boulaksil M, van Stuijvenberg L et al. In calcineurin-induced cardiac hypertrophy expression of Nav1.5, Cx40 and Cx43 is reduced by different mechanisms. *J Mol Cell Cardiol* 2008 September;45(3):373-84.

(50) Bourajjaj M, Armand AS, da Costa Martins PA et al. NFATc2 is a necessary mediator of calcineurin-dependent cardiac hypertrophy and heart failure. *J Biol Chem* 2008 August 8;283(32):22295-303.

(51) Noorman M, Hakim S, Kessler E et al. Remodeling of the cardiac sodium channel, connexin 43, and plakoglobin at the intercalated disk in patients with arrhythmogenic cardiomyopathy. *Heart Rhythm* 2012 November 23.

(52) Khoo MS, Kannankeril PJ, Li J et al. Calmodulin kinase II activity is required for normal atrioventricular nodal conduction. *Heart Rhythm* 2005 June;2(6):634-40.

(53) Boulaksil M, Winckels SK, Engelen MA et al. Heterogeneous Connexin43 distribution in heart failure is associated with dispersed conduction and enhanced susceptibility to ventricular arrhythmias. *Eur J Heart Fail* 2010 September;12(9):913-21.

(54) van Rijen HV, Eckardt D, Degen J et al. Slow conduction and enhanced anisotropy increase the propensity for ventricular tachyarrhythmias in adult mice with induced deletion of connexin43. *Circulation* 2004 March 2;109(8):1048-55.

(55) Royer A, van Veen TA, Le Bouter S et al. Mouse model of SCN5A-linked hereditary Lenegre's disease: age-related conduction slowing and myocardial fibrosis. *Circulation* 2005 April 12;111(14):1738-46.

(56) Stein M, Boulaksil M, Jansen JA et al. Reduction of fibrosis-related arrhythmias by chronic renin-angiotensin-aldosterone system inhibitors in an aged mouse model. *Am J Physiol Heart Circ Physiol* 2010 August;299(2):H310-H321.

(57) Jansen JA, Noorman M, Musa H et al. Reduced heterogeneous expression of Cx43 results in decreased Nav1.5 expression and reduced sodium current that accounts for arrhythmia vulnerability in conditional Cx43 knockout mice. *Heart Rhythm* 2012 April;9(4):600-7.

(58) Stein M, van Veen TA, Remme CA et al. Combined reduction of intercellular coupling and membrane excitability differentially affects transverse and longitudinal cardiac conduction. *Cardiovasc Res* 2009 July 1;83(1):52-60.

(59) van Veen TA, Stein M, Royer A et al. Impaired impulse propagation in Scn5a-knockout mice: combined contribution of excitability, connexin expression, and tissue architecture in relation to aging. *Circulation* 2005 September 27;112(13):1927-35.

(60) Jansen JA, Noorman M, Musa H et al. Reduced heterogeneous expression of Cx43 results in decreased Nav1.5 expression and reduced sodium current that accounts for arrhythmia vulnerability in conditional Cx43 knockout mice. *Heart Rhythm* 2012 April;9(4):600-7.

(61) Jansen JA, van Veen TA, de Jong S et al. Reduced Cx43 expression triggers increased fibrosis due to enhanced fibroblast activity. *Circ Arrhythm Electrophysiol* 2012 April;5(2):380-90.

Summary (English)

Abbreviations:
CaMKII: calcium/calmodulin-dependent protein kinase II
LTCC: L-type calcium channel
NCX: sodium/calcium exchanger
RyR: ryanodine receptor
TdP: torsade des pointes arrhythmias

Background

The sole purpose of the heart is to pump blood through the body. In order to achieve this, it is important that the rhythm is regular and controlled, that the contractile force of the muscle is sufficient, and that the macrostructure of the heart retains the right balance, between for example wall thickness and volume of the lumen. Remarkably, calcium plays an important role in all these processes.

The rhythm of the heart is controlled via electrical impulses. After the sinus node fires spontaneously, the electrical signal is transported via intracellular coupling. In addition, the electrical signal in the (ventricular) cardiomyocytes themselves, the action potential, is a strictly controlled cellular depolarization. Calcium is involved in the start of the electrical impulse, as the upstroke in the sinus node is calcium-dependent, and in the ventricular cardiomyocytes, influx of positively charged calcium ions has a net depolarizing effect during the action potential. In these two ways calcium is involved in the electrical signal, and thus the control of the cardiac rhythm.
In cardiac contraction, calcium is also important, as the cellular contraction is directly controlled by intracellular calcium concentrations. Is the concentration high, contraction occurs, while the subsequently lowered concentration leads to relaxation. As an increase in intracellular calcium concentration is initially induced by voltage sensitive calcium channels, calcium is also responsible for excitation-contraction coupling.
Concerning the structural balance of the heart, a number of calcium-sensitive signalling pathways exist, including the CaMKII and calcineurin pathways. As such, calcium is also importation in regulating cardiac hypertrophy.

This multifaceted role of calcium in the heart is mirrored in what happens in pathology. Disturbances in calcium balance can lead to disruption of the cardiac rhythm (arrhythmias). It can also lead to reduced force of contraction or insufficient relaxation, and it can induce unbalanced, pathological hypertrophy. Because of this, calcium is a tempting target. But, as it is involved in multiple facets of cardiac function, it will also be difficult to influence one calcium-dependent factor, without influencing other calcium-sensitive processes as a side effect. In this thesis, we nonetheless tried to target cardiac calcium while preventing negative secondary effects.

Contents of this thesis

In the preface (**chapter 1**) of this thesis the rationale behind this research is explained, based on both the potential and difficulties of targeting calcium in the heart.
Next, in **chapter 2**, physiological excitation-contraction and impulse propagation is described, as well as an overview of arrhythmogenic mechanisms, with an emphasis on triggered activity. It describes the close interconnectivity of calcium in excitation (influx of positively charged calcium ions) and contraction (as contraction is controlled by intracellular calcium concentrations). Furthermore, in the case of triggered activity calcium can be involved in two ways. 1) An influx of calcium via the LTCC as a window current can lead to early afterdepolarizations, and 2) RyR leakage can give rise to delayed afterdepolarizations. Additionally, removal of calcium out of the cell via the NCX gives rise to a net depolarizing current, exacerbating the effect of calcium leak on the membrane potential. These mechanisms both give a theoretical framework on why calcium block can be anti-arrhythmic, as well as why it can reduce the force of cardiac contraction.

Chapter 3 goes beyond the cellular level to investigate organ-wide electrophysiology, as well as the effect of structural changes. It provides an in depth insight into physiological ventricular remodeling, especially focused on the chronic atrioventricular block dog. This model is characterized by compensated hypertrophy; a growth of the heart without significant decrease in cardiac pump function. This is in contrast to decompensated hypertrophy, which tends to deteriorate into heart failure, even when there is a latent period with temporary preservation of cardiac function. Remarkably, calcium-dependent signalling pathways (calcineurin and CaMKII) seem to be mainly involved if the hypertrophy is decompensated, while their role seems minor in physiological hypertrophy. The Akt pathway displays an inverse role and is probably dominant in compensated situations.
The AV-block dog with compensated hypertrophy is used in the following **chapter (4)** to investigate the effects evoked by CaMKII in long-QT dependent arrhythmias and hypertrophy. It turned out that CaMKII was more activated in the atrioventricular block dog, without alterations in its absolute expression level. This could potentially explain why this model has compensated instead of decompensated hypertrophy. After all, in the case of heart failure both activity and expression of CaMKII are elevated. Additionally, this dog model shows no increased activity of calcineurin, also involved in pathological hypertrophy, but Akt phosphorylation is present, which is more indicative of physiological hypertrophy. Nonetheless, in this model electrophysiological characteristics are disturbed leading to an increased propensity for arrhythmias. Despite the lack of increased expression, acute inhibition of CAMKII proved to be an effective anti-arrhythmic strategy.
In **chapter 5** we used a mouse model with chronic CaMKII inhibition and decompensated hypertrophy accompanied by a reduced conduction velocity and arrhythmias. Surprisingly, chronic inhibition of CaMKII did not prevent arrhythmias, although CaMKII inhibition was able to rescue conduction velocity. This has some interesting repercussions for the perceived mechanism of arrhythmias in this model, but the full details are not yet resolved.
In the subsequent **chapter (6)** another mouse model with decompensated hypertrophy and arrhythmias was used as the research tool, but in this case the hypertrophy was due to overexpression of constitutively active calcineurin, another calcium-sensitive signalling protein. It was hypothesized that CaMKII inhibition would reduce the hypertrophy and the arrhythmias, as CaMKII has been reported to be involved in both. However, in this model, adding chronic inhibition of CaMKII appeared ineffective in rescuing conduction velocity related parameters like connexin and sodium channel expression, the implication being that CaMKII effects on hypertrophy and arrhythmias are situation dependent.
The thesis switches to other calcium-related targets in **chapter 7**, where it is described what the most profound negative side-effect is of verapamil and other calcium blockers: reduced cardiac contractility. This could be especially detrimental in heart failure, as the contraction reserve of the heart is then already diminished. Our reaction to *Milber*g *et al.* pointed out that the arrhythmic effectiveness of verapamil was indeed plausible, but that hemodynamic problems were not taken into account.
Based on that we hypothesized that we could circumvent this undesired side effect by using a combined blocker of the LTCC and NCX, as is described in **chapter 8**. Theoretically, blocking LTCC would diminish (arrhythmic) depolarizing inflows of calcium, but a simultaneous NCX inhibition would slow calcium extrusion and as such save intracellular calcium concentrations. This hypothesis indeed appeared to be correct. In vitro, SEA-0400 (a combined LTCC and NCX blocker) did not decrease intracellular calcium concentrations. It did increase diastolic calcium levels slightly, but in vivo diastolic ventricular blood pressure seemed not to be influenced. Most importantly, systolic pressure was preserved with SEA-0400, and it appeared able to prevent afterdepolarizations in vitro, and TdPs in vivo. Compared to verapamil, SEA-0400 was just as anti-arrhythmic, but with only negligible

effects on left ventricular pressure. This holds promise as a potential anti-arrhythmic drug that may be superior to class IV anti-arrhythmics.
Chapter 9 comprises the general discussion, which tried to resolve a number of unexplained findings, and provides a general appraisal of the combined data of the thesis.

Conclusion

The original hypothesis was partially confirmed. It was indeed possible to acutely inhibit cardiac arrhythmias by targeting calcium without an accompanied reduction of cardiac contractile force. However, chronic modulation of calcium-sensitive signalling pathways to prevent pathological cardiac remodelling turned out to be not very straightforward. In all, it must be concluded that the acute effects of calcium in excitation and contraction are sufficiently known to warrant optimism in the near future, but that the chronic effects of calcium-dependent signalling will need further elucidation before reliable predictions can be made regarding the effects of targeting these pathways.

Samenvatting (Nederlands)

Afkortingen:
CaMKII: Calcium/calmoduline afhankelijke kinase II
LTCC: L-type calcium kanaal
RyR: ryanodine receptor
NCX: natrium/calcium exchanger

Achtergrond

In essentie is het hart een holle spier die bloed door het lichaam pompt. Als het hart ontspannen is kan bloed de holte binnenstromen. Als de spieren in de hartwand dan vervolgens samentrekken wordt het bloed uit het hart gepompt, en via de bloedvaten door het hele lichaam verspreidt.
Wil het hart zijn functie goed kunnen uitvoeren dan zijn een aantal zaken van groot belang: Ten eerste moet het ritme regelmatig zijn. Ten tweede moet het hart met voldoende kracht samentrekken, en ten derde moet het hart de juiste grootte hebben met de correcte verhoudingen.

Opvallend genoeg speelt calcium een rol in al deze zaken. Hierbij moet calcium niet gezien worden als een vaste stof, zoals de mineralen in botten, maar als in vloeistof opgeloste deeltjes die calciumionen worden genoemd.
Het ritme van het hart wordt bepaald door een elektrisch signaal. Dit begint in een specifiek gedeelte van het hart, de sinusknoop, en verspreidt zich vervolgens razendsnel over het hart zodat elke hartspiercel tegelijkertijd geactiveerd wordt. Als de cellen elektrisch negatief geladen zijn, zijn ze in rust. Als ze positief geladen raken worden ze geactiveerd. Calcium speelt hierbij een rol omdat het een positief geladen molecuul is. Als de cellen geactiveerd raken stroomt het calcium de cellen in en helpt dus mee om de cellen positief te laden.
Calcium is ook betrokken bij de samentrekkingskracht, omdat de moleculen in elke spiercel die verantwoordelijk zijn voor de samentrekking reageren op calcium. Is de concentratie calcium in de cel hoog, dan trekt de cel samen. Is de concentratie laag, dan ontspannen de cellen.
Als laatste is calcium ook betrokken bij de groei van het hart. Er zijn eiwitten in elke hartspiercel die het signaal kunnen geven om te gaan groeien. Sommige van deze signaaleiwitten zijn gevoelig voor calcium en op deze manier beïnvloedt calcium dus de grootte van het hart.

Op dezelfde manier kan calcium dus ook betrokken zijn bij het ontstaan van ziektes van de hartspier. Raakt de calciumhuishouding verstoort dan kan dat leiden tot hartritmestoornissen, tot een te zwakke samentrekking van de hartspier, of tot ongezonde groei van het hart. Calcium is immers betrokken bij al deze processen.
Het is om deze reden dat deze thesis zich richt op de rol van calcium in het hart. Doordat het diverse rollen vervult in het hart is calcium een aantrekkelijk doelwit om aan te pakken bij ziekte, maar tegelijkertijd maakt deze multifunctionele rol ingrijpen erg complex. Immers, ingrijpen op één van deze calcium afhankelijke zaken kan lijden tot bijeffecten in andere processen die ook afhankelijk zijn van calcium. Aantrekkelijk dus, maar complex.

Deze thesis

In de preface (voorwoord, **hoofdstuk 1**) wordt de rationale van dit onderzoek uitgelegd, dat gebaseerd is op zowel de mogelijk- als moeilijkheden van het ingrijpen op calcium in het hart. Vervolgens wordt in **hoofdstuk 2** uitgebreid beschreven welke rol calcium speelt bij het hartritme en de activiteit en samentrekking van hartspiercellen. Ook wordt uitgelegd hoe hartritmestoornissen kunnen ontstaan, met uiteraard een focus op calcium hierin. Het hoofdstuk beschrijft hoe de effecten van calcium op excitatie (influx van calcium) en

contractie (aangezien dat reageert op intracellaire calciumconcentraties) aan elkaar gelinkt zijn. Daarnaast beschrijft het dat calcium op twee manieren kan leiden tot 'triggered activity' (extra slagen), namelijk doordat er via het LTCC calcium de cel binnenstroomt, of doordat er lekkage uit het sarcoplasmatisch reticulum plaatsvindt, een intracellulaire opslagplaats voor calcium. Op beide manieren kan de cel positiever geladen raken, en dus aanleiding geven tot activatie en ritmestoornissen. Deze combinatie van mechanismen is de theoretische achtergrond op basis waarvan wij menen dat het aanpakken van calciumstromen anti-arritmisch kan zijn, maar ook waarom het de samentrekking van het hart kan verminderen.
Hoofdstuk 3 gaat verder dan het cellulaire niveau om de elektrofysiologie van het hart op orgaanniveau te bekijken. Het gaat specifiek in op fysiologische ventriculaire remodelering, specifiek in het hondenmodel met chronische atrioventriculaire block. De hypertrofie in dit model is gecompenseerd, dat wil zeggen, de groei van het hart gaat niet samen met een afname van pompfunctie. Dit in tegenstelling tot gedecompenseerde hypertrofie, waar de groei van het hart overgaat in hartfalen, zelfs als er een latente periode is waarin de hartfunctie tijdelijk op niveau blijft.
Calcium-afhankelijke signaaltransductie (waarin o.a. de eiwitten calcineurine en CaMKII belangrijk in zijn) is voornamelijk betrokken bij gedecompenseerde hypertrofie, terwijl de rol bij fysiologische hypertrofie miniem is. De Akt afhankelijke signaaltransductie daarentegen wordt vooral gekoppeld aan fysiologische veranderingen. Het lijkt erop dat het verschil tussen gecompenseerde en gedecompenseerde hypertrofie al terug te vinden is op het niveau van intracellulaire signaaltransductie.
Het hondenmodel met atrioventriculaire block wordt in het volgende **hoofdstuk (4)** gebruikt om de effecten van CaMKII te onderzoeken op hypertrofie en (lange QT afhankelijke) ritmestoornissen. Alhoewel CaMKII wel meer geactiveerd bleek te zijn in dit model, was de eiwitexpressie niet omhoog. Dit kan mogelijk verklaren waarom de hypertofie in deze honden fysiologisch is, aangezien bij hartfalen activatie én expressie van CaMKII verhoogd zijn. Daarnaast is calcineurine ook niet verhoogd in dit model, maar Akt is wel meer actief, wat dan weer gekoppeld wordt aan fysiologische groei. Ondanks de niet verhoogde expressie van CaMKII, bleek het inhiberen van CaMKII effectief in het terugbrengen van ritmestoornissen in dit model, zowel *in vitro* als *in vivo*.
In **hoofdstuk 5** hebben we een muizenmodel gebruikt, maar nu met gedecompenseerde hypertrofie welke tot stand komt door via een chirurgische ingreep het uitpompen van het bloed te belemmeren. Deze ingreep leidt na verloop van tijd tot hartfalen en daarnaast wordt dit model gekarakteriseerd door een verlaagde geleidingssnelheid over het hart en een toegenomen gevoeligheid voor ritmestoornissen.
Tot onze verrassing bleek langdurige CaMKII remming wel de geleidingssnelheid te verbeteren, maar niet in staat te zijn tot het voorkomen van ritmestoornissen. Dit heeft een aantal interessante gevolgen wat betreft het mechanisme achter deze ritmestoornissen, maar de details zijn nog niet volledig bekend.
In **hoofdstuk 6** gebruikten we opnieuw een muizenmodel met gedecompenseerde hypertrofie, maar in dit geval werd dit veroorzaakt door overexpressie van calcineurine, een calcium-afhankelijk signaaltransductie eiwit waarvan gedacht wordt dat het tot hartfalen kan leiden. De hypothese was dat chronische CaMKII remming in dit model zowel de hypertrofie als de ritmestoornissen zou tegenhouden, aangezien CaMKII geacht wordt betrokken te zijn bij allebei. Echter, deze remming bleek geen enkel effect te hebben op moleculaire parameters waarvan gedacht wordt dat ze betrokken zijn bij afname van de geleidingssnelheid, zoals de expressie van connexines (formeren gap junctions) en het eiwit dat het natriumkanaal formeert.
Het proefschrift gaat een iets andere kant op in **hoofdstuk 7**, waarin beschreven wordt wat de negatieve bijeffecten zijn van verapamil en andere remmers van LTCC, namelijk dat ze de

samentrekkingskracht van het hart verminderen. Vooral in het geval van hartfalen, wanneer de hartfunctie al sterk verminderd is, kan dit negatieve gevolgen hebben. In onze reactie op *Milberg et al.* gaven we aan dat wat ons betreft het anti-arritmische effect van deze middelen niet ter discussie stond, maar dat in de praktijk hemodynamiek de belemmerende factor zou zijn.
Dit leidde tot de hypothese dat de negatieve bijwerkingen van LTCC blokkade zouden kunnen voorkomen door zowel de LTCC als de NCX te remmen, zoals beschreven is in **hoofdstuk 8**. De gedachte hierachter was dat het blokkeren van de LTCC de depolariserende (en dus pro-arritmische) effecten van calciuminstroom zou verminderen, maar doordat NCX blokkade de uitstroom van calcium remt, de intracellulaire calciumconcentratie (en dus contractie) op peil zou kunnen blijven. Gebaseerd op onze resultaten lijkt dit correct te zijn. *In vitro* had toediening van SEA-0400 (een middel dat zowel LTCC als NCX remt) geen verlaging van de calciumconcentratie tot gevolg. Tijdens diastole was er zelfs een kleine verhoging van calcium. Dit leek echter geen effecten te hebben *in vivo*, waar de bloeddruk in het hart binnen normale waarden bleef. Dit in tegenstelling tot verapamil dat aanleiding gaf tot een 15% reductie in maximale ventriculaire bloeddruk. Beide middelen waren volledig anti-arritmisch. In andere woorden: SEA-0400 is net zo effectief tegen ritmestoornissen als verapamil, maar zonder de hemodynamiek in het hart te verstoren. Dit maakt het mogelijk superieur aan andere calciumremmers (klasse IV anti-arritmica), als dit in verdere studies ook zo blijkt te zijn.
Hoofdstuk 9, tot slot, is de algemene discussie die de resultaten van alle bovenstaande hoofdstukken bij elkaar brengt.

Conclusie

Oorspronkelijk dachten we dat we door calcium aan te pakken zowel slechte groei van het hart konden voorkomen en ook ritmestoornissen konden verminderen zonder de samentrekkingskracht van het hart aan te pakken. Uit dit onderzoek is echter gebleken dat dit slechts ten dele waar is. Ja, we hebben aangetoond dat het goed mogelijk is om calcium aan te pakken en via die manier ritmestoornissen te verminderen, zelfs zonder afname van de hartspierkracht, maar het verminderen van ziektegroei van het hart is op deze manier nog niet gelukt. We moeten concluderen dat er nu voldoende bekend is aangaande de rol van calcium om ritmestoornissen effectief aan te pakken, maar dat de rol van calcium in de groei van het hart nog niet genoeg doorgrond is om er nu al betrouwbaar op in te kunnen grijpen.

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De student die mij het meeste heeft bijgestaan is **Lars**. Tijdens je laudatio heb ik gezegd dat je een perfect evenwicht in stand hield tussen zelfstandigheid en samenwerking. Dat die samenwerking ook effectief en prettig was heb ik geloof ik niet gezegd, maar het is wel zo. En je bent de koning van de Western Blot, natuurlijk.
Naast Lars heeft ook **Helen** een belangrijke bijdrage geleverd. Zeker hoofdstuk twee is jouw kindje, maar ook in de immunohistologische resultaten zit jouw werk. Ik bewonder je extroverte enthousiasme, evenals je uitgesproken voorkeur voor allerhande (en soms onwelriekende) kazen. De volgende keer hoef je me echter niet noodzakelijk deelgenoot te maken van je fascinatie voor (soms obscure) Olympische sporten ;-).
Zoals Jet de baas van de OK is, is **Marien** dat voor de geïsoleerde cardiomyocyte metingen. Een groot aantal van de figuren in dit proefschrift die single cell action potentials laten zien zijn gebaseerd op data die jij gegenereerd hebt op lange avonden in het elektrofysiologielab. Verder is een avond actiepotentialen meten uiteraard niet compleet zonder een uitgebreide dosis straaljagertrivia.
Leonie, naar het einde van dit project heb ik kunnen profiteren van jouw uiterst professionele labkunde. Dank daarvoor. En natuurlijk voor een prettige combinatie van gezelligheid met soms een heel klein tikje eigenwijsheid ;-) .

Op het gebied van de calcineurine muizen ben ik ook dank verschuldigd aan een groot aantal mensen. Ten eerste natuurlijk aan **Marti**, bedankt dat ik in dit boeiende project kon meedraaien. Verder zijn we de laatste paar maanden kamergenoten geweest, waar je me de voordelen van een opgeruimd bureau en een stabiel gemoed hebt laten zien. En ik heb een boel nieuwe dingen over aquaria geleerd.
Hanneke, via dit project heb ik ook direct met jou samengewerkt. Ik was danig onder de indruk van de enorme hoeveelheden werk die jij in korte tijd wist te verzetten. Ik ben benieuwd of je van plan bent om verder te gaan met dit project. Ook verwacht ik natuurlijk nog alle mooie verhalen over Australië te horen.
Naast Hanneke, heb ik ook met **Bart** samengewerkt via dit project. Wat is er mooier dan een goed gelukte CaMKII blot? Meer dan dat echter, moet ik je danken voor de goede sfeer die jij wist te creëren in het moleculaire lab en natuurlijk de helpende hand die je altijd bereid was te bieden.
Magda, when Roel was gone, and I was too incompetent to perform a proper Langendorff perfusion, you stepped in to help me. It was critical at that moment, and it is much appreciated. Equally, if not more, appreciated are the micro-lessons Portuguese (through wind, snow, or rain, while riding home after work) you gave me. Obrigado! I will also not forget the guided tour you have given me and Thom in Lisboa. I was mightily impressed by your driving skills, not so much by your historical knowledge ☺ .
Ook **Harold** ben ik dankbaar wat betreft de Langendorff perfusies. Toen zelfs Magda's kennis (en de batterij) tekort schoot was jij er om ons te helpen. Bovendien is het vertederend om te zien hoe gelukkig jij word van een opgewekte ritmestoornis.
Hoofdstuk 7, de editorial over verapamil, was een initiatief van **Marcel**. En succesvol kunnen we wel zeggen. De tijd dat ik jou als kamergenoot heb gehad heeft me geleerd dat het altijd essentieel is om wetenschappelijke (en zelfs de niet wetenschappelijke) ideeën correct te formuleren. Je bent een scherp wetenschapper, en ik hoop dat ik daar een klein beetje van heb kunnen overnemen.
Thom, ook jouw naam staat op die editorial, en terecht. Maar meer dan dat ben ik je dankbaar voor alle hulp die je mij geboden hebt met betrekking tot het CAVB model. Als ik iets niet begreep, of simpelweg de kennis niet paraat had, was jij altijd bereid om het mij rustig uit te leggen. Daarnaast beweert Tonny dat jij en ik nogal goed zijn in discussiëren. Naar mijn mening is dat een compliment.
Helemaal aan het eind van dit project, terwijl ik de laatste data aan het uitwerken was, bleek **John** bereid om mij op weg te helpen, ook al werkte hij niet meer bij ons. Dat was dus pure vriendelijkheid, en daar ben ik je dankbaar voor. Bovendien is mijn leven niet meer hetzelfde nu de Likkende Jezus te Paard zich aan mij geopenbaard heeft.
Hiroki, it was you who first noticed that CaMKII might inluence conduction velocity. I sincerely hope this will mean our work will culminate in a splendid article. I also have to thank you of course for the Manju baking, or should I thank Noriko for this? We also spend quite a few conferences together, so I must also thank you for not snoring ☺ . Your enthusiasm for European cities is contagious, by the way.
Aangezien een groot gedeelte van dit werk onderdeel was van de Leducq transatlantic CaMKII alliance, kwam ik ook in contact met de Proteomics afdeling. **Albert** en **Arjen**, bedankt voor dit inkijkje in jullie keuken. Niet dat ik nu veel begrijp van proteomics, maar mijn fascinatie is wel toegenomen.

De sfeer bij Medische Fysiologie is altijd goed geweest, en ik kan niets anders dan mijn collega's daarvoor beddanken.

Tonny, jouw kamer was veruit de gezelligste! En ik ga de gesprekjes missen die afwisselend heel banaal of persoonlijk konden zijn, en alles daar tussenin. (en mijn excuses voor alle taalfouten in dit boekje waar je je ongetwijfeld aan zal ergeren).
Rianne, je bent slecht voor mijn productiviteit, maar zeer geslaagd als koffiemaatje. Ik doe hier geen uitspraken over je gevoel voor humor. Je mag zelf bepalen of dat een compliment is. Samen naar huis fietsen heeft ons inderdaad iets dichter bij het oplossen van een aantal wereldproblemen gebracht, **Malin**. Graag wijs ik er overigens op dat een discussie met zijn tweeën wordt gevoerd. Succes met wereldburger zijn!
Christian, de gesprekken met jou hebben ongetwijfeld mijn horizon verbreed. Kunst en Cultuur. Ik zal proberen om wat meer literatuur te lezen.
Die enkele keer dat ik echt heel vroeg op het werk was, wist ik altijd dat ik op de aanwezigheid van één iemand kon rekenen: **Rosanne**. Bedankt voor alle heel-erg-in-de-vroege ochtend gesprekjes.
It was always nice to know that there was at least one PhD student besides me who knew what CaMKII meant. Siddarth, your ambition is impressive, although I find your talent for partying also very praiseworthy. And no, you will never get me drunk.
Sofieke, soms krijg ik het idee dat je alles weet over TdPs en STV dat er te weten valt. Zeer indrukwekkend. We moeten ook nog steeds een autoritje doen!
Albert, ik vind het passend dat jij begint nu ik stop. Het voelt een beetje alsof je er altijd bij bent geweest, aangezien we een tijdje (al weer lang geleden) de studentenkamer hebben gedeeld.
Er zijn maar weinig mensen die zoveel plezier uitstralen als jij **Sanne**. Volgens mij is het onmogelijk om lang boos of chagrijnig te zijn als jij er bij bent.
Het is mede dankzij jou dat ik bij Medische Fysiologie terecht ben gekomen, **Maria**. Tijdens de Master heb je mij de mogelijkheid gegeven om een variatie aan stages uit te zoeken, waaronder op fysiologisch gebied. Ik heb medelijden met jouw computer, maar niet met je studenten. Die zitten wel goed bij jou.
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Mijn voormalig kamergenootje **Maartje** moet ook nog even genoemd worden, natuurlijk. Al was het alleen maar vanwege de prachtige witte Vespa met bijpassende roze schoenen!
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Mijn familie heeft me ook altijd steun gegeven. Meer dan ik hier op zou kunnen noemen. Niettemin is het dankwoord niet af zonder specifiek **mijn ouders** te noemen. En **Monique & Rutger, Bets & Henk**, en **Oma** natuurlijk. Dank voor alle liefde en aanmoediging. Maar wederom, waarom hier noemen wat a) toch niet helemaal in woorden valt te vatten, en b) over veel meer gaat dan enkel een proefschrift?

List of Publications

Combined Na+/Ca2+ Exchanger and L-Type Calcium Channel Block as a Potential Strategy to Suppress Arrhythmias and Maintain Ventricular Function.
Bourgonje VJ, Vos MA, Ozdemir S, Doisne N, Acsai K, Varro A, Sztojkov-Ivanov A, Zupko I, Rauch E, Kattner L, Bito V, Houtman M, van der Nagel R, Beekman JD, van Veen TA, Sipido K, Antoons G.
Circ Arrhythm Electrophysiol. 2013 Mar 20. [Epub ahead of print]

Relevance of calmodulin/CaMKII activation for arrhythmogenesis in the AV block dog.
Bourgonje VJ, Schoenmakers M, Beekman JD, Nagel RV, Houtman MJ, Miedema LF, Antoons G, Sipido K, de Windt LJ, van Veen TA, Vos MA.
Heart Rhythm. 2012 Jul 27

Verapamil as an antiarrhythmic agent in congestive heart failure: hopping from rabbit to human?
Stams TR*, Bourgonje VJ*, Vos MA, van der Heyden MA.
Br J Pharmacol. 2012 May;166(2):554-6. doi: 10.1111/j.1476-5381.2011.01818.x

Ventricular Electrical Remodeling in Compensated Cardiac Hypertrophy
Vincent J.A. Bourgonje, Toon A.B. van Veen, Marc A. Vos
Electrical Diseases of the Heart, 2nd ed. (Book chapter)

Abstracts:

Combined Na/Ca exchanger and L-type calcium channel block by SEA-0400 suppresses Torsade de pointes arrhythmias with maintained haemodynamics
V.J.A. Bourgonje, M.A. Vos, S. Ozdemir, K. Acsai, N. Doisne, R. Van Der Nagel, H.D.M. Beekman, T.A.B. Van Veen, K.R. Sipido and G. Antoons
Cardiovasc Res (2012) 93 (suppl 1): S92-S127. doi: 10.1093/cvr/cvr336

The anti-arrhythmic effect of CaMKII inhibition in calcineurin overexpressing hearts is independent of Cx43, Cx40, Nav1.5, and fibrosis remodeling
A.J.A. Raaijmakers, V.J.A. Bourgonje, G.J.M. Kok, A.A.B. Van Veen, M.E. Anderson, M.A. Vos and M.F.A. Bierhuizen
Cardiovasc Res (2012) 93 (suppl 1): S92-S127. doi: 10.1093/cvr/cvr336

Discrepancy between acute and long-term effects of the calmodulin/CaMKII/calcineurin pathway on arrhythmogenesis in the CAVB dog
V.J.A. Bourgonje, M. Schoenmakers, M.J. Houtman, H.D.M. Beekman, R. van der Nagel, L.J. de Windt, A.A.B. van Veen, M.A. Vos
29 Aug 2010 Basic and translational science European Society of Cardiology

Discrepancy Between Acute and Long-Term Effects of the Calmodulin-Camkii-Calcineurin Pathway on Arrhythmogenesis in the CAVB Dog
Vincent J.A. Bourgonje, Marieke Schoenmakers, Jet D.M. Beekman, Roel van der Nagel, Leon J. de Windt, Toon A.B. van Veen, Marc A. Vos
Heart Rhythm Volume 6, Issue 11, Pages 1693-1694, November 2009

curriculum vitae

Vincent Bourgonje was born on May 26th, 1985 in Apeldoorn, The Netherlands. He graduated (Atheneum) at the Veluws College Walterbosch in Apeldoorn in 2003. The following September he started the Bachelor Biomedische Wetenschappen (Biomedical Sciences) at Utrecht University. After graduation, this was followed in 2006 by the biomedical Master Biology of Disease.
Internships included research on the pre-fusion coronaviral spike protein, and the influence of thyroid hormone on endothelial cells and angiogenesis (University Utrecht, Faculty of Veterinary Medicine, department of Virology and Anatomy & Physiology, respectively).
An additional internship at Medical Physiology (UMC Utrecht) on the arrhythmogenic effects of CaMKII lead directly to a PhD position at the same department, starting in 2008.
Under the supervision of Prof. Dr. Marc Vos and Dr. Toon van Veen the project 'Calcium in the heart: a tempting target' came to fruition in 2013 with this thesis. At the 14th of may 2013 the results of this project will be defended in public.