Arrhythmia mechanisms and effects of exercise training in CPVT and post-infarction heart failure

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Oslo, November 2022, *Tore Kristian Danielsen*

Table of Contents

1 Abbreviations

AP = Action potential
AV = Atrioventricular
CaM = Calmodulin
CAMKII = Ca^{2+}/Calmodulin-dependent kinase type II
CSQ = Calsequstrin 2
CICR = Ca^{2+} induced Ca^{2+} release
CPVT = Catecholaminergic polymorphic ventricular tachycardia
DAD = Delayed afterdepolarization
EAD = Early afterdepolarization
ECC = Excitation contraction coupling
ECG = Electrocardiogram
EF = Ejection fraction
ET = Exercise training
FKBP12.6 = FK-binding protein12.6 (a.k.a. Calstabin 2)
HF = Heart failure
ICD = Implantable cardioverter defibrillator
ISO = Isoprenaline
JCTN = Junctin
LTCC = L-type Ca^{2+} channel
MI = Myocardial infarction
NCX = Na^{+}/Ca^{2+} exchanger
PKA = Protein kinase A
PMCA = plasma membrane Ca^{2+} ATPase
PVC = Premature ventricular complex
ROS = Reactive oxygen species
RYR = Ryanodine receptor
SCD = Sudden cardiac death
SED = Sedentary
SERCA2 = SR Ca^{2+} ATPase type 2
SOICR = Store overload-induced Ca^{2+} release
SR = Sarcoplasmic reticulum
TECRL = Trans-2,3-enoyl-CoA reductase like
TRDN = Triadin
VF = Ventricular fibrillation
VT = Ventricular tachycardia

2 Summary in Norwegian

2.1 Arytmimekanismer og effekten av trening ved CPVT og postinfarkt hjertesvikt

Pasienter med hjertesvikt og pasienter med den arvelige sykdommen katekolaminerg polymorf ventrikkeltakykardi (eng.: Cathecholaminergic Polymorphic Ventricular Tachycardia, CPVT) har til felles at de kan få hjertestans grunnet arytmi forårsaket av forandringer i et viktig protein i hjertet kalt ryanodinreseptoren (RyR). RyR er viktig i reguleringen av kalsium i hjertemuskelcellene, og dermed for hjertets elektriske aktivitet og pumpefunksjon. I hver hjertesyklus frigis kalsium fra det sarkoplasmatiske retikulum til cytosol via RyR. Kalsium binder seg til troponin i sarkomerene, og fører til sammentrekning av cellen. Kalsium blir etter en kontraksjon raskt fjernet fra cytosol og pumpet tilbake til sarkoplasmatisk retikulum. Frislippet av kalsium skal normalt foregå i systolen, mens fjerning fra cytosol skjer i diastole. Begge prosesser er kontrollert av en rekke reguleringsmekanismer. Ved endringer i RyRs funksjon, slik man kan se ved CPVT og hjertesvikt, kan man imidlertid få frislipp av kalsium fra det sarkoplasmatiske retikulum i diastolen. Denne typen frislipp av kalsium kan føre til sene etterdepolariseringer. Sene etterdepolariseringer kan føre til unormal elektrisk aktivering av kardiomyocytter, og i verste fall til hjertestans og plutselig død.

I mitt PhD arbeid har jeg undersøkt mekanismer for arytmi ved CPVT, og effekten av trening på tendensen til arytmi ved CPVT og hjertesvikt. Sammen med mine medforfattere har jeg samlet data fra både pasienter, rotter og mus til disse undersøkelsene. Forsøkene ble utført med genmodifiserte mus med en mutasjon i RyR (R2474S), som man finner hos en del av pasientene med CPVT, og med rotter med hjertesvikt etter hjerteinfarkt. I en av studiene ble det også inkludert data fra pasienter med CPVT som følges ved Oslo universitetssykehus Rikshospitalet, samt datamodellering av kalsiumhåndtering i kardiomyocytter. Det ble høstet vev fra hjertene til mus og rotter som ble brukt til proteinanalyser. Kalsiumhåndtering ble undersøkt i kardiomyocytter isolert fra muse- og rottehjerter.

I den første artikkelen i avhandlingen samlet vi data fra pasienter med CPVT. Slike pasienter har økt risiko for hjertestans ved fysisk eller emosjonelt stress, det vil si situasjoner med økt adrenalinfrigjøring. Pasienten gjennomførte en standard sykkeltest med gradvis økende motstand. Individuell pulsterskel for arytmi ble registrert som puls målt ved første ventrikulære ekstrasystole i bigemini, koblete ventrikulære ekstrasystoler eller ventrikulær takykardi under sykkeltesten. Våre data viste at pasientene hadde en individuell pulsterskel

for utvikling av ventrikulære ekstrasystoler. Denne terskelen ble sammenliknet med resultater fra økning av pulsen ved pacemakerstimulering hos tre pasienter med implanterbar hjertestarter med atrieledning. Disse tre pasienten ble stimulert på en puls som var 5-10 høyere en terskelpulsen målt på sykkeltest, og når dette ble gjort i hvile førte det ikke til arytmi. Data fra forsøk med kardiomyocytter fra CPVT-mus bekreftet at den viktigste faktor for å øke risikoen for rytmeforstyrrelser er adrenalinstimulering, men viste samtidig at høy hjerterytme i seg selv er medvirkende årsak til økt kalsiumfrislipp som kan føre til arytmi. Datamodellering viste et komplisert samspill mellom reopptak av kalsium i sarkoplasmatisk retikulum og variasjon i RyRs aktivitet, som avgjør om man får diastolisk frislipp av kalsium i diastole som kan føre til arytmi.

I den andre artikkelen undersøkte vi om intervalltrening påvirker kalsiumhåndtering i kardiomyocytter fra CPVT-mus, og tendensen til arytmi i disse musene. Musene gjennomførte en treningsprotokoll med intervalltrening på tredemølle i to uker, og ble sammenliknet med mus som ikke trente. Arbeidet viste at intervalltrening gir økt maksimalt oksygenopptak i mus med CPVT, og stabiliserer RyR på en måte som beskytter mot arytmi. Et av proteinene som regulerer RyR er Ca^{2+}/Calmodulin-dependent kinase type II (CaMKII). En effekt av trening i CPVT var mindre CaMKII-avhengig fosforylering av RyR. Dette stabiliserte RyR, og ga dermed mindre frislipp av kalsium i diastolen, og færre episoder med sene etter-depolariseringer av kardiomyocytter. Færre sene etterdepolariseringer reduserte tendensen til ventrikulære ekstrasystoler: CPVT-mus som gjennomførte trening, hadde færre episoder med hjertearytmi målt med EKG under stress, sammenlignet med utrente mus med CPVT. Disse resultatene kan tyde på at intervalltrening kan minske risikoen for arytmier ved CPVT.

I den tredje artikkelen undersøkte vi hvordan trening påvirker hjertefunksjonen og kalsiumhåndteringen i kardiomyocytter ved postinfarkt hjertesvikt i en rottemodell. Rottene gjennomførte en treningsprotokoll med intervalltrening på tredemølle i fem uker, og ble sammenliknet med rotter som ikke trente. Som et mål for treningseffekt viste vi at dette økte maksimalt oksygenopptak i trente rotter, sammenlignet med utrente. Når vi undersøkte hjerter fra disse dyrene, viste det seg at trening gir en bedring av kalsiumhåndteringen i hjertet. I kardiomyocytter fra trente rotter med hjertesvikt var det mindre frislipp av kalsium i diastolen sammenlignet med kardiomyocytter fra utrente rotter. Ved hjertesvikt kunne vi ikke vise samme endring i fosforylering-graden av RyR som vi målte i trente hjerter fra mus med CPVT, men vi viste at trening normaliserer nivået av beta-adrenerge reseptorer i rotter med

hjertesvikt. Hvorvidt dette er forklaringen på stabiliseringen av RyR vil kreve ytterligere forskning å avklare.

Resultatene i denne avhandlingen viser at trening kan stabilisere RyR og gi mindre frislipp av kalsium i diastolen ved både CPVT og hjertesvikt. Dette indikerer at trening har potensiale til å være en terapeutisk strategi mot arytmier som skyldes endret RyR-funksjon. Trening kan ha potensiale til å redusere risikoen for arytmier og hjertestans ved CPVT og hjertesvikt, men mer forskning er nødvendig for å avklare hvordan dette kan utnyttes på en trygg måte.

3 List of papers

Paper 1

Arrhythmia initiation in catecholaminergic polymorphic ventricular tachycardia type 1 depends on both heart rate and sympathetic stimulation

Tore K. Danielsen, Mani Sadredini, Ravinea Manotheepan, Jan M. Aronsen, Karina Hougen, Ole M. Sejersted, Ivar Sjaastad, Mathis K. Stokke

PLoS One 2018 Nov 6; 13 (11): e0207100

Paper 2

Exercise training prevents ventricular tachycardia in CPVT 1 due to reduced CaMKII-dependent arrhythmogenic Ca^{2+} release

Ravinea Manotheepan, Tore K. Danielsen, Mani Sadredini, Mark E. Anderson, Cathrine R. Carlson, Stephan E. Lehnart, Ivar Sjaastad, Mathis K. Stokke

Cardiovasc Res 2016; 11: 295-306

Paper 3

Exercise training stabilizes RyR2-dependent Ca^{2+} release in post-infarction heart failure

Tore K. Danielsen, Mani Sadredini, Ravinea Manotheepan, Jan M. Aronsen, Michael Frisk, Marie H. Hansen, Kjetil W. Andressen, Karina Hougen, Finn O. Levy, William E. Louch, Ole M. Sejersted, Ivar Sjaastad, Mathis K. Stokke

Front Cardiovasc Med. 2021 Jan 25; 7: 623922

4 Introduction

> *"When the heart is diseased, its work is imperfectly performed: the vessels proceeding from the heart become inactive, so that you cannot feel them… if the heart trembles, has little power and sinks, the disease is advanced and death is near."*

This quote from the Ebers Papyrus from ancient Egypt pre-dating 1500 B.C., is thought to be the earliest record that describes sudden cardiac death (SCD) caused by arrhythmia, (1) i.e. sudden arrhythmic death, the most dramatic outcome of the conditions studied in this thesis. According to current guidelines from the European Society of Cardiology, sudden death and SCD is defined as

> *"(…) a non-traumatic, unexpected fatal event occurring within 1 hour of the onset of symptoms in an apparently healthy subject. If death is not witnessed, the definition applies when the victim was in good health 24 hours before the event. Sudden cardiac death is used when a congenital, or acquired, potentially fatal cardiac condition was known to be present during life, or autopsy has a cardiac or vascular anomaly as the probable cause of the event or no obvious extra-cardiac causes have been found by post-mortem examination and therefore an arrhythmic event is a likely cause of death." (2)*

Even with the tremendous increase in the understanding of mechanisms leading to arrhythmias that has been gained in the last decades, SCD due to arrhythmias is still a major global health problem, (2-4) that occurs in 50-100 per 100.000 persons every year, (5, 6) although with variation between reports due regional differences and different definitions of SCD. The term cardiac arrest is often used interchangeably with SCD. According to the Norwegian Cardiac Arrest Registry, 4213 out-of-hospital cardiac arrests occurred in Norway in 2020, which gave an incidence of 78 cardiac arrests per 100.000 persons per year. (7)

Tachyarrhythmias are a more common cause of SCD than bradyarrhythmias (app. 70 vs 30 %), with ventricular tachycardia (VT), that ultimately degenerates to ventricular fibrillation (VF), by far the most common type of tachyarrhythmia. (8) This thesis focuses on conditions and mechanisms that underlie ventricular tachyarrhythmias. The dominant conditions that lead to VT vary with age: While inherited cardiac disease is the most common

cause in persons below 30 years, coronary heart disease and heart failure (HF) is the dominant cause in older persons. (9) This thesis describes efforts to understand mechanisms and potential therapeutic strategies for VT caused by the rare genetic disease catecholaminergic polymorphic ventricular tachycardia type 1 (CPVT1), and VT occurring as a result of HF after myocardial infarction (MI).

4.1 Cellular electrophysiology and normal cardiac function

Each heartbeat has a systolic (contraction) and a diastolic (relaxation) phase, in which pumping of blood and filling of the cardiac chambers, respectively, occurs. Underlying this process is electric activity (10): Each normal heartbeat is initiated by electrical activity in the sinoatrial (SA) node. At rest, a voltage difference can be measured across the membrane of all cardiomyocytes, i.e. the membrane potential, with the cytosolic side being negative compared to the extracellular side of the membrane; the membrane is said to be polarized. This is the result of large differences in positive and negative charges on either side of the cell membrane; the outside has a large surplus of positive charges mainly due to Na^+, while the inside has a large surplus of negative charges due to negatively charged proteins. Additionally, the cell inside holds a high concentration of K^+, and as K^+ channels are the only ion channels open at rest, the membrane potential at rest is highly dependent on the K^+ gradient across the membrane. The differences in concentrations of Na^+ and K^+ is maintained by the Na^+/ K^+ ATPase, ensuring active efflux of Na^+ and influx of K^+.

Pacemaker cells in the SA node have a capacity to generate a spontaneous drop in the transmembrane voltage difference, i.e. a depolarization. Activity in the autonomic nervous system controls the frequency of this behavior. The depolarization in pacemaker cells of the SA node spreads to other cells in the atria and reaches the atrioventricular (AV) node. Propagation of the depolarization slows down in the AV node, which allows for filling of the ventricles, before further propagation through the Bundle of His and Purkinje fibers results in depolarization of the ventricular cardiomyocytes, and to contraction. The rapid spread of depolarization through the conduction system ensures a coordinated and efficient contraction of the ventricles. (10)

Electrical activity leads to contraction in a process referred to as the excitation-contraction coupling (ECC), which is tightly coupled to variations in the membrane potential. (11) This variation occurs in phases controlled by different ion currents, and the variation through each heartbeat is called the action potential (AP). In ventricular cardiomyocytes, the

initiating event is a local depolarization of the cell membrane that spreads to a cell-wide activation of voltage-dependent Na^+ channels with influx of Na^+ and a full depolarization of the cardiomyocyte (Phase 0 of the AP). In this phase, the membrane potential even tends to reverse, with the inside turning positive compared to the outside. The depolarization activates voltage-gated transient outward K^+ channels which leads to outward flux of K^+ that counteracts the depolarizing effect of the Na^+ current (phase 1). With a short delay, phase 1 is followed by influx of Ca^{2+} through voltage gated L-type Ca^{2+} channels in phase 2. Influxing Ca^{2+} binds to and activates ryanodine receptors (RyR2) in the sarcoplasmic reticulum (SR), and a sudden and rapid release of Ca^{2+} occurs. This process is called Ca^{2+} induced Ca^{2+} release (CICR), and is structurally secured by the close proximity of LTCC and RyR2 in subcellular units called dyads. (11, 12)

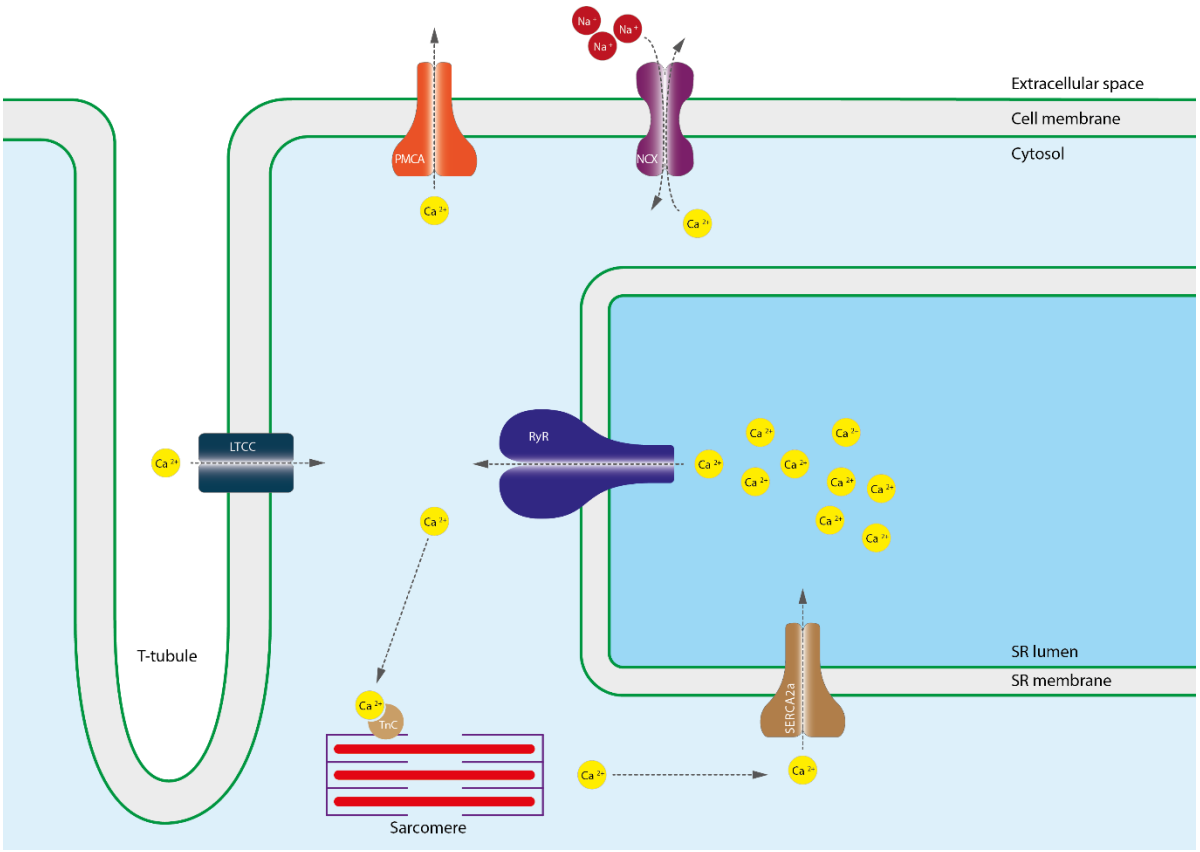

Figure 1. Illustration of a cardiomyocyte with important Ca^{2+} handling ion channels. Arrows indicate direction of Ca^{2+} flux.

Release of Ca^{2+} from the SR results in a rapid rise in the Ca^{2+} concentration in the cytosol, from approximately 100 nM to 1µM (13). This leads to increased Ca^{2+} binding to troponin C in the contractile apparatus of the cardiomyocytes, and cross bridging of actin and myosin with subsequent contraction. (14) The rise and fall in cytosolic Ca^{2+} concentration is termed the Ca^{2+} transient, and the amplitude and length of the Ca^{2+} transient together with the Ca^{2+} sensitivity of the contractile apparatus determines the force of contraction. (11) For relaxation, Ca^{2+} in the cytosol is rapidly moved back into the SR by the cardiac form of the SR Ca^{2+} ATPase (SERCA2), and out of the cell by the Na^{+}/Ca^{2+} exchanger (NCX), while a small amount is removed by the plasma membrane Ca^{2+} ATPase (PMCA). Ca^{2+} removal by NCX tend to depolarize the cell membrane by extruding 1 Ca^{2+} ion in exchange for 3 Na^{+} ions, resulting in a net influx of positively charged ions. (15, 16) In humans, about 70% of cytosolic Ca^{2+} is removed by SERCA2, while in mice and rats SERCA2 contributes to more than 90% of cytosolic Ca^{2+} removal. (11)

With a further delay from initiation of the AP, and towards the end of phase 2, voltage gated K^{+} channels are activated. Subsequent K^{+} outflow leads to repolarization of the cell membrane in phase 3 of the AP, and brings the cell membrane back to its initial state in phase 4. (17)

4.2 Regulation of sarcoplasmic reticulum Ca^{2+} release and reuptake

The ECC is initiated and controlled by the AP, but importantly regulated by the activity of Ca^{2+} handling proteins in the cardiomyocytes, which is subject to tight regulation by a number of mechanisms. At the heart of this activity is the interplay between RyR2 and SERCA2. RyR2 is a large 565 kd protein forming a tetramer consisting of four RyR2 proteins. In total, the *Ryr2* gene has 105 exons, while the protein consists of 4976 amino acids. (18) Each RyR2 subunit is divided into two cytoplasmic domains called the N-terminal domain and the central domain, and a third transmembrane region, which is the C-terminal domain. (19) The cytoplasmic N-terminal is the domain regulating the activity of the ion channel by interacting with RyR2 regulatory proteins. (16, 20, 21) The RyR2 protein tetramers in the SR are organized into release units or clusters, which are thought to be functional units that consist of multiple RyR2 tetramers. Ca^{2+} release from one cluster is named a Ca^{2+} spark, and graded Ca^{2+} release in the Ca^{2+} transient is possible partly due to variations in the amount of Ca^{2+} released in a spark, partly due to variations in the number of RyR2 clusters recruited for Ca^{2+} release. (22, 23)

RyR2 release Ca^{2+} upon binding of Ca^{2+} from the LTCC, in a process called Ca^{2+}-induced-Ca^{2+}-release. (12) It has long been recognized that the FK-binding protein12.6 (FKBP12.6, also named Calstabin 2) stabilizes the closed state of RyR2, and thereby prevents diastolic SR Ca^{2+} leak. (24, 25) However, other proteins also regulate RyR2 activity. (26-28) Among the most studied are calmodulin (CaM), calsequstrin-2 (CSQ), junctin (JCTN), and triadin (TRDN). CASQ2 binds Ca^{2+} inside the SR as a Ca^{2+} storing protein. (26) RyR2 is linked to CASQ2 via TRDN and JCTN, and SR Ca^{2+} regulates the action of RyR2 through its effects on this interaction, and also by direct Ca^{2+} binding to RyR2. (27) CaM binds CASQ2 to RyR2, and CASQ2, TRDN and JCTN forms a complex that is found close to RYR2. (28) The exact roles of TRDN and JCTN in the RYR2 complex are still unknown, but both TRDN and JCTN are thought to bind CASQ2 to RYR2. (28, 29) TRDN is also found to sensitize RYR2 and thereby enhance SR Ca^{2+} release. (30, 31)

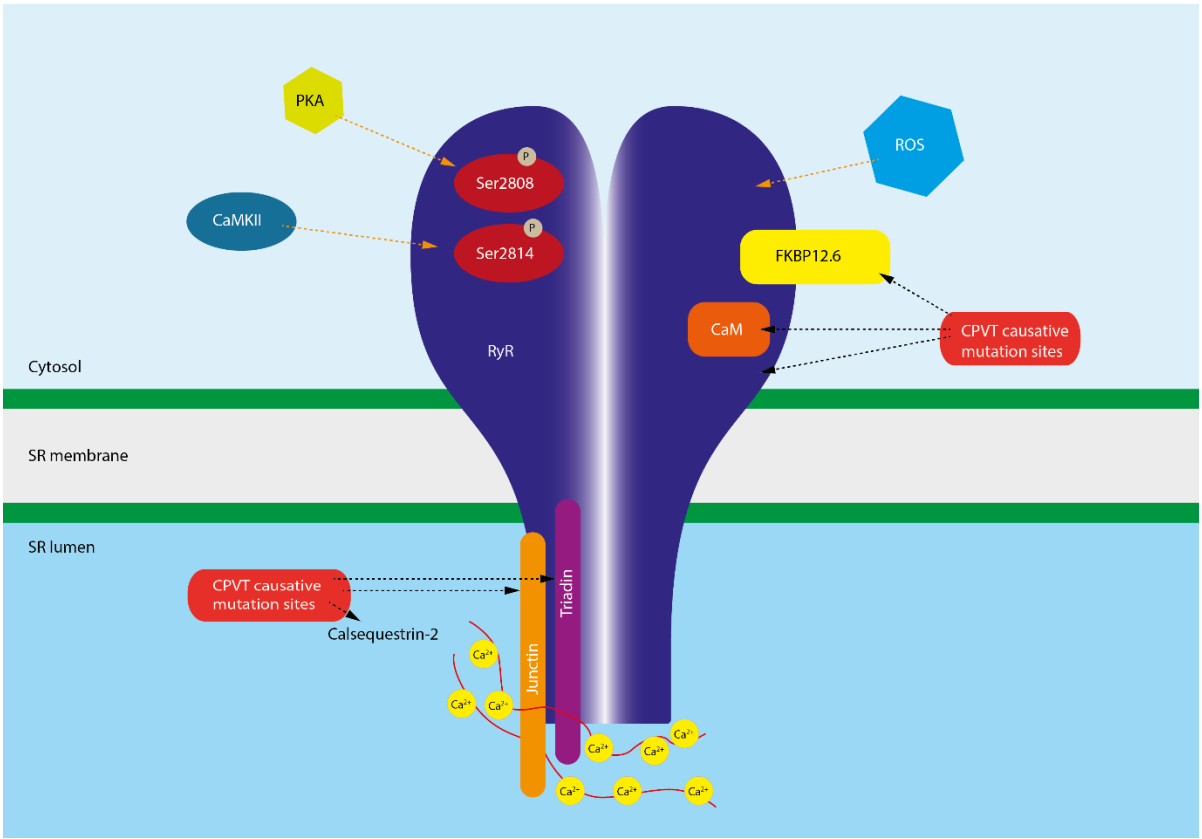

Fig. 2 Illustration of RyR2 with important RyR2 regulators in the cardiomyocytes.

RyR2 activity is importantly regulated by beta-adrenoceptor-dependent signaling. Catecholamine binding to beta-adrenoceptors at the surface of the cardiomyocytes leads to

increased enzymatic production of cyclic AMP (cAMP), which activates protein kinase A (PKA). (11) It has lately been shown that inhibition of PKA abolishes the effect of the beta-agonist ISO on both cardiac and cardiomyocyte contractility, and also inhibits the normal increase in HR during beta-adrenoceptor stimulation. (32) PKA phosphorylates RyR2 at Ser2808 in small rodents and humans. (33) The mechanistic details of the effects of this RyR2 phosphorylation are subject of debate, but most agree that RyR2 Ser2808 phosphorylation by PKA leads to increased RYR2 sensitivity for cytosolic Ca^{2+}, resulting in a higher open probability of RyR2 and increased SR Ca^{2+} release. (24, 34) One proposed mechanism has been dissociation of FKBP12.6 from RyR2, leading to increased SR Ca^{2+} release. (24) Provided that sufficient Ca^{2+} is available in the SR, the increased sensitivity results in more Ca^{2+} being released in each heartbeat, with more cross-binding and increased contractile force. Increased Ca^{2+} sensitivity of RyR2 normally coincides with increased SR Ca^{2+} content during beta-adrenoceptor activation, as PKA activation also leads to increased SERCA2 activity through phosphorylation of phospholamban, and increased influx of Ca^{2+} trough LTCC. (11) The end result of PKA phosphorylation of RYR2 has been opposed by others, (35) with no effect on RyR2 function after removal of FKBP12.6. (36)

Another important regulator of the ECC is Ca^{2+}/calmodulin-dependent protein kinase type II (CaMKII), which regulates RyR2 function through phosphorylation at RyR2 Ser2814. (37) Phosphorylation of this site is low in absence of beta-adrenoceptor stimulation and pacing; only 7 % of maximum, compared to 57 % at the PKA-dependent phosphorylation site Ser2808 in rat cardiomyocytes. (38, 39) Activation of CaMKII is in itself a complex process: It is activated by binding of Ca^{2+} and CaM, but can also be auto-phosphorylated at Thr287, which prolongs its activation beyond the immediate presence of Ca^{2+}. (40) Furthermore, oxidation (41), glycosylation (42), and nitrosylation (43) have all been found to be potential contributors to CaMKII activation, and indirectly to RyR2 dysregulation in diseased hearts. The role of PKA and CaMKII dependent phosphorylation of RyR2 will be further discussed below in the specific contexts of CPVT1 and HF.

4.3 RyR2 dysfunction, triggered activity and ventricular tachyarrhythmias

Three principle mechanisms can generate VT: Reentry, abnormal automaticity or triggered activity. (44) Triggered activity causes abnormal impulse generation which can cause arrhythmias. This thesis concerns triggered arrhythmias caused by delayed afterdepolarization (DAD), i.e. depolarization of the cell membrane in phase 4 of the AP.

Specifically, the work presented in this thesis focus on DADs caused by RyR2 dysfunction. Triggered activity also comprises early afterdepolarizations (EADs), i.e. depolarisations in phase 2 or 3 of the AP. Although some studies indicate that EADs and DADs in some situations share underlying mechanisms, (45) EADs mainly occur in situations with prolonged AP and as a consequence of reactivation of the LTCC. EADs are therefore not the focus of this thesis.

RyR2 dysfunction can result in SR Ca^{2+} release in phase 4 of the AP. The probability and size of this Ca^{2+} release depend on the concentration of Ca^{2+} in the SR and the Ca^{2+} sensitivity of RyR2. (46-48) The mechanism underlying the SR Ca^{2+} release is reflected in the term "store overload-induced Ca^{2+} release" (SOICR) which has been used for this type of Ca^{2+} release. (49) Ca^{2+} released locally from one RyR2 cluster due to SOICR increases cytosolic Ca^{2+} concentration in the dyad. The increase of cytosolic Ca^{2+} activates NCX, which extrudes 1 Ca^{2+} in exchange for 3 Na^{+}. This exchange therefore results in a net positive transient inward current, which tends to depolarize the cell membrane. In addition, some of the Ca^{2+} released from the SR by RyR2 diffuses to neighboring RyR2 clusters and initiates further SR Ca^{2+} release through CICR. Further diffusion-release-diffusion events result in a propagating Ca^{2+} wave. As more Ca^{2+} is released from the SR, more Ca^{2+} is exchanged for Na^{+} by NCX, depolarizing the cardiomyocyte, and resulting in a DAD. (50)

Triggered activity can lead to PVCs and promote tachyarrhythmias. VT occurs either as a result of repetitive bursts of triggered activity in groups of cardiomyocytes, or from reentry circuits activated by premature beats resulting from triggered activity. (51) For DADs to trigger ectopic activation of the myocardium, DAD-triggered APs must occur simultaneously in a large number of cardiomyocytes. In one study using data from a rabbit model of HF in a computational model, the number of cells needed to trigger macroscopic ectopic activity, i.e. a ventricular ectopic beat recognized in the ECG, was 4,189. Interestingly, this number was much higher in the model with data from healthy hearts (57,906). (52) Based on the large number of cells that need to be activated, arrhythmias caused by triggered activity might seem unlikely. However, triggered activity most often occur in situations with SR Ca^{2+} overload, for example during catecholamine stimulation of the heart. (51) Such stimulation both increases the likelihood of SOICR and synchronizes the occurrence of such events in groups of cells in the myocardium, thereby increasing the probability for DADs and ectopic activity. (53) In the following paragraphs, the importance of triggered arrhythmia in CPVT and HF will be described in more detail.

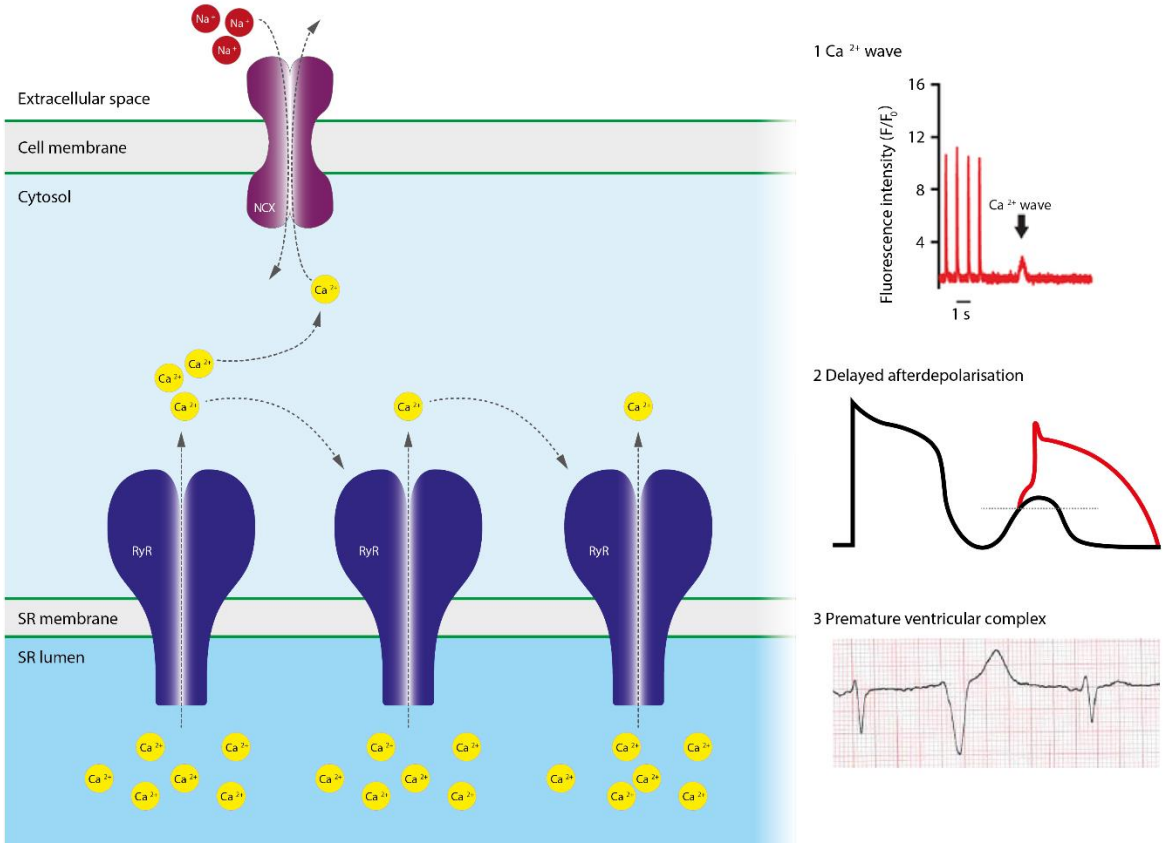

Figure 3: Illustration of a cardiomyocyte with spontaneous SR Ca^{2+} release through RyR2. Spontaneous SR Ca^{2+} release can activate neighboring RyR2 and NCX. This can be measured as a Ca^{2+} wave (1), and lead to delayed afterdepolarisation (2) and premature ventricular complex recorded in ECG (3).

4.4 Catecholaminergic polymorphic ventricular tachycardia – a model disease for triggered arrhythmias

The first clinical description of CPVT was made by Reid *et al.* in 1975. (54) They described bidirectional VT in a 6-year-old girl, without evidence of structural heart disease, and noticed that the arrhythmia seemed to appear during physical or emotional stress. Later reports have confirmed this typical phenotype, although phenotype expression varies in terms of age of debut and severity. (55-58) Patients with CPVT have a normal ECG at rest, and develop ventricular arrhythmias in situations associated with increased sympathetic activity and increased heart rate. (59) Typically, the phenotype is revealed during cardiopulmonary exercise testing. (60) As the heart rate increase, an increasing number of PVCs occurs, typically followed by PVC in bigeminy and ventricular tachycardia if exercise continues. The

VT often has the characteristic bidirectional pattern, in which the QRS axis alternates from beat to beat. (60)

Current guidelines provide diagnostic criteria for CPVT, with one of the following criteria needed to confirm the diagnosis (61):

1. CPVT **is diagnosed** in the presence of a structurally normal heart, normal ECG and unexplained exercise or catecholamine-induced bidirectional VT, polymorphic ventricular beats or VT in individuals < 40 years of age.
2. CPVT **is diagnosed** in patients (index case or family member) who have a pathogenic mutation.
3. CPVT **is diagnosed** in family members of a CPVT index case with a normal heart who manifests exercise-induces PVCs or bidirectional / polymorphic VT.
4. CPVT **can be diagnose** in the presence of a structurally normal heart and coronary arteries, normal ECG, and unexplained exercise or catecholamine-induced bidirectional VT, polymorphic ventricular beats or VT in individuals > 40 years of age.

In approximately 60 % of patients with CPVT, the disease phenotype can be ascribed to a mutation in one of six genes: *RyR2,* (18, 62) *CSQ,* (63, 64) *KCNJ2* gene, (65) *CaM1, CaM3* (calmodulin), (66, 67) *TRDN* (Triadin), (68) and *TECRL* (Trans-2,3-enoyl-CoA reductase-like gene). (69) Based on the involved genes, CPVT is divided into subtypes 1 to 5: CPVT type 1 (CPVT1) is the most abundant form and is caused by mutations in the *RyR2,* inherited in an autosomal dominant pattern. (60) Mutations in *CSQ, KCNJ2, CaM* and *TRDN* cause the much more rare CPVT subtypes 2-5. Lately, it has been disputed if mutation in *KCNJ2* is causative for CPVT, or if conditions due mutations in this gene should be categorized under the Andersen-Tawil syndrome. (70)

Mutations associated with CPVT1 cause RyR2 dysfunction, which leads to increased leak of SR Ca^{2+} in diastole, DADs and VT. (24, 71, 72) On a molecular level, different mechanisms can explain how *RyR2* mutations cause the protein dysfunction that leads to increased Ca^{2+} leak from the SR: Some *RyR2* mutations might lower binding affinity between RyR2 and FKBP12.6. This can lead to dissociation between the two proteins when RyR2 is phosphorylated by PKA. (24) In one mouse model, increased FKBP12.6 binding to RyR2 prevented stress-induced VT and SCD. (73) *RyR2* mutations might also lower the threshold for SOICR, by enhancing RyR2 luminal SR Ca^{2+} activation, (49) or disrupt interactions between the N-terminal and the C-terminal of the RyR2 protein. (74) It has been shown

clinically that patients with *RyR2* mutations in the C-terminal are more prone to episodes of VTs compared to RyR2 mutation in the N-terminal. (58) Studies of a mouse model have suggested that the *RyR2* R2474S mutation causes a disrupted interaction between the N-terminal and the central domain of RyR2, and thereby decreases the threshold SR Ca^{2+} content for RyR2 activation. This alteration leads to CPVT-associated RyR2 activity with increased Ca^{2+} spark frequency in presence of beta-adrenoceptor stimulation, but also increased Ca^{2+}spark frequency even without such stimulation (75).

Ventricular arrhythmias in CPVT result from triggered activity. (76) Increased diastolic SR Ca^{2+} leak has been thoroughly characterized in cardiomyocytes with CPVT causing mutations, (76) and triggered activity has been convincingly demonstrated in a number of cellular and mouse models with human mutations, as well as by measurements of monophasic APs in patients with CPVT. (71, 73, 77-79) However, the mechanism for how SR Ca^{2+} leak on a cellular level causes VT in structurally normal hearts of patients with CPVT, is not entirely clear. A complex mechanism involving alternating triggered activity between cells in two different foci has been suggested to explain the characteristic bidirectional VT. (80) A mouse model with *RyR2* R4496C mutation also found the Purkinje fibers to be the origin of the VT in CPVT. (81) Ablation of the right ventricle endocardium induced right bundle branch block and converted the bidirectional VT into a monomorphic VT in the same model. (81) The mechanism behind this is not fully understood, but one clue might come from studies by Herron *et al*. who showed that Purkinje cells with a *RyR2* R4496C mutation develop SR Ca^{2+} release at a greater rate and greater amplitude than working ventricular cardiomyocytes with the same *RyR2* mutation. (82)

Another explanation for VT in CPVT is that DADs resulting from SR Ca^{2+} release creates electrical heterogeneities in the myocardium that effectively provides a dynamic substrate for varying local functional reentry circuits. (83) It has been proposed that VT in CPVT originates from the epicardium, thereby increasing the transmural dispersion of repolarization, and leading to focal reentry which involves a limited myocardial mass. (84) Although, the number of cells needed to reach the threshold for focal excitation is lower in diseased hearts compared to healthy myocardium, one could speculate that the number of cells reaching this threshold in patients with CPVT might still be sufficient. (52) Importantly, the Purkinje-dependent and reentry-dependent mechanisms are not mutually exclusive.

Mutations of *TRDN, CaM* and *CSQ* can also cause CPVT. These three proteins all bind to RyR2 and modulate the function of RyR2. The mechanisms for how mutations in *CSQ* lead to increased SR Ca^{2+} leak are still focus for debate and research. (76, 85) The

mechanisms for CPVT caused by mutations in *TRDN* could be weakened or decreased binding of CSQ to RyR2, which leads to increased SR Ca^{2+} leak and thereby increased risk of arrhythmias. (86) Mutations in *CaM* also result in increased propensity for Ca^{2+} sparks, Ca^{2+} waves and DADs. (67) The underlying mechanism involves loss of regulation of RyR2. A canine model of HF exhibited loss of CaM regulation of RyR2 and increased SR Ca^{2+} leak. (87) The *RyR2* R2474S mutation have been claimed by some authors to cause lower binding affinity between RyR2 and CaM in cardiomyocytes stimulated with cAMP, and thereby increased RyR2 activity. (88) Correction of CaM binding to RyR2 in cardiomyocytes with this mutation normalized Ca^{2+} handling. (89)

The drugs used for initial treatment of CPVT are beta-adrenoceptor antagonists, (2, 61) with nadolol considered the drug of choice by many clinicians. (56, 90) Recently, a multicenter study supported that non-selective beta blockers, of which nadolol is one, should be the preferred choice in children with CPVT. (91) If beta-adrenoceptor antagonists are insufficient to prevent arrhythmias, flecainide is recommended. (61) Early reports showed that even during treatment with beta-adrenoceptor antagonists, 46 % of CPVT patients experienced PVCs, VT or SCD, (60) while with addition of flecainide such events occurred in only 2 out of 13 patients. (92) Another study of 29 patients with CPVT showed partially or complete suppression of ventricular arrhythmias for 8 and 14 patients, respectively, when flecainide was added to a beta-adrenoceptor antagonist. (93) Flecainide has lately been shown to inhibit RyR2 directly and reduce SR Ca^{2+} release in a mouse model of CPVT harboring a mutation in CSQ, (94) even if the mechanism of flecainide is debated. (95, 96) Patients that continue to experience serious arrhythmic events despite optimal medical treatment are recommended an implantable cardioverter defibrillator (ICD). (61) However, implantation of ICDs in patients with CPVT is a difficult choice as adequate ICD-treatment might trigger a burst of catecholamine release and arrhythmic storm. (97, 98) Another option is left cardiac sympathetic denervation which has been used with success in a limited number of patients. (99, 100)

4.5 Triggered arrhythmias in heart failure

HF is a clinical syndrome consisting of cardinal symptoms with breathlessness, ankle swelling, and fatigue that may be accompanied by signs such as elevated jugular venous pressure, pulmonary crackles, and peripheral edema. (101) The clinical syndrome is due to a structural and/or functional abnormality of the heart that results in elevated intracardiac

pressures and/or inadequate cardiac output at rest and/or during exercise. (101) The now classic definition formulated by Braunwald that "HF is a pathophysiological state in which an abnormality of cardiac function is responsible for the failure of the heart to pump blood at a rate commensurate with the requirements of the metabolizing tissues" (102) is still valid. Although clinically, HF is categorized as HF with reduced ejection fraction (HFrEF), i.e. EF $\leq$40%, HF with mildly reduced EF (HFmrEF), i.e. EF 41- 49%, and HF with preserved ejection fraction (HFpEF), i.e. EF $\geq$50%. (101) For the following part of this thesis, HF refers to HFrEF, unless otherwise explicitly stated. Measurements of biomarkers are also used as a diagnostic tool to identify patients with HF. (103) The level of B-type natriuretic peptides are related to the risk of HF development, (104) and measurement of plasma concentration of natriuretic peptides is recommended as diagnostic test for patients with HF symptoms. (101) A lot of work has been performed to investigate new possible biomarkers as diagnostic tools for HF. One proposed biomarker is related to this thesis. Secretoneurin has been proposed as biomarker of dysfunctional Ca^{2+} handling in HF and a prognostic tool to asses risk of arrhythmias. (105, 106)

HF continues to have a poor prognosis, with a 12-month all-cause mortality of 17 % for hospitalized HF patients. (107) The underlying cause for HF development is often multifactorial and involves ischemic heart diseases, with known risk factors such as hypertension, diabetes type 2, hyperlipidemia, and obesity. In addition to ischemic heart diseases, valvular abnormalities, arrhythmias or conducting system defects, and diseases of the peri- or myocardium, including cardiomyopathies, are important causes for HF development. The mechanism underlying the development of myocardial dysfunction in each of these conditions is complex, and many aspects still remain to be elucidated.

This thesis concerns ventricular tachyarrhythmias in HF. Although reentry is probably the most common cause for sustained VT in HF, abnormal automaticity and triggered activity can act as triggers for reentry episodes and probably cause non-sustained and even sustained episodes independent of classic reentry circuits. (44) However, the evidence supporting that sustained VT can be caused by triggered activity is mostly based on animal models and extrapolations from mechanistic understanding, and direct evidence is missing due to methodological challenges with separating the different mechanisms in patients.

The basic mechanisms underlying triggered activity in HF, specifically mechanisms underlying DADs, and the possibility for ET to affect this mechanisms, are the focus of paper 3 in this thesis. The paper focuses on mechanisms for DADs in cardiomyocytes from rats, but similar mechanisms have been shown in models of HF in many other species, including

canine models, (108) and in ventricular tissue from patients with HF. (109) Several factors contribute to an increased propensity for triggered activity in HF: Increased sympathetic activity is a frequent finding in HF models, (110) and thought to be a key factor in disease development and progression. (111) In the context of this thesis, the effect of increased beta-adrenoceptor stimulation on Ca^{2+} handling in the cardiomyocytes is especially important, and the role of the downstream signaling molecules PKA and CaMKII is a major focus. The effect of chronically increased beta-adrenoceptor activity on these key effectors alters Ca^{2+} handling through altered RyR2 function in cardiomyocytes during HF, which contributes to SR Ca^{2+} leak. (34, 112) PKA has important short and long-term effects in this process: In the acute and short term, PKA is the key effector of beta-adrenoceptor stimulation, activating CaMKII, and leads to an increase in systolic Ca^{2+} release through increased influx of Ca^{2+} through the LTCC, increased uptake of Ca^{2+} to SR through SERCA2, and phosphorylation of RyR2. (113) These effects are mediated by phosphorylation of target proteins, and phosphorylation of RyR2 is believed to interrupt the binding to FKBP12.6, as shown in cardiomyocytes from human and dogs with HF (34). On the other hand, chronic PKA-dependent hyperphosphorylation of RyR2 Ser2808 has been proposed to contribute to a reduced Ca^{2+} transient amplitude, and thereby contractile impairment and arrhythmias in HF. (114) The proposed mechanism is that chronic PKA-dependent phosphorylation of RyR2 Ser2808 increases diastolic SR Ca^{2+} leak, resulting in SR Ca^{2+} depletion. (34, 115) The mechanism for this effects is probably also dissociation of FKBP12.6 from RyR2, as supported by findings in both human tissue (34, 116) and in animal models of HF. (117, 118) Mutations in the FKBP12.6-binding regions of RyR2 associated with CPVT1 also seems to support the importance of the RyR2-FKB12.6 interaction. (24, 119) However, the importance of the RyR2-FKBP12.6 interaction has been disputed. (72, 120) Briefly, some studies have found that PKA-dependent phosphorylation of RyR2 Ser2808 does not affect Ca^{2+} homeostasis after myocardial infarction. (121) Ai *et al.* found increased PKA-dependent phosphorylation of RyR2 Ser2808, but that only increased CaMKII-dependent phosphorylation of RyR2 Ser2814 contributed to increased SR Ca^{2+} leak and lower SR Ca^{2+} load in HF. (112) Similar results have been found in a rabbit model of heart failure, where PKA inhibition did not alter SR Ca^{2+} leak. (122) The debate continues, and it seems that further studies are needed to establish the full understanding of PKA-mediated effects on RyR2, and the consequence for cardiomyocyte function in health and disease.

Increased sympathetic activity in HF also activates CaMKII. (41) CaMKII phosphorylates RyR2 Ser2814, which leads to increased open probability and thereby

enhanced SR Ca^{2+} leak. (123-125) Guo T *et al.* showed that the CaMKII-dependent phosphorylation of RyR2 Ser2814 increased SR Ca^{2+} leak, by longer Ca^{2+} sparks and more frequent propagating SR Ca^{2+} release events, which they propose to be an explanation for the increased propensity for triggered arrhythmias in HF. (126) Increased SR Ca^{2+} leak due to CaMKII-dependent phosphorylation of RyR2 Ser2814 has also been found by others. (112, 122, 127)

Among the numerous processes occurring in HF, increased RyR oxidation by reactive oxygen species (ROS) might also play a role, and is of importance for the focus of this thesis. Oxidation of RyR2 has been proposed to increase its activity and contribute to increased SR Ca^{2+} leak during HF, although the exact mechanism is not clear. (128) FKBP12.6 binding to RyR2 was found to be lowered by ROS oxidation by Shan *et al.*, (117) but others have found FKBP12.6 binding to RyR2 to be normal in similar conditions, and suggested that the alterations caused by ROS oxidation of RyR2 is due to altered binding to CaM. (129, 130)

An increase in the late sodium current might also play a role for triggered activity in HF, (131) through a secondary increase in diastolic Ca^{2+} and activation of CaMKII. (132) Sodium accumulation with subsequent Ca^{2+}-dependent activation of CaMKII and DADs was shown in an animal model of pressure overload-induced HF, in which DADs were prevented by CaMKII inhibition. (133) Further support for sodium accumulation ending with CaMKII activation has been provided by computational modeling. (134)

Paradoxically, reduced SERCA2, found in many HF models and tissue from patients with HF, (135) might also contribute to increased propensity for triggered activity during beta-adrenoceptor stimulation. The mechanism probably involves secondary effects on other Ca^{2+} handling proteins. (136)

NCX abundance is also altered, although there are reports on both increased and lower NCX function in HF. (137-139) An increase in NCX abundance will increase the transient inward current and DADs resulting from Ca^{2+} waves. (137) Combined with reduced I_{K1}, this has been proposed as an important part of the explanation for increased propensity for triggered activity in HF. (140) A recent publication expanded this model of general mechanisms for triggered activity in HF to a comprehensive system involving Na^{+}-Ca^{2+}-CaMKII-ROS-I_{NaL}-interactions. (141)

Perturbations in cellular and subcellular structure might also be important for triggered activity in HF: T-tubule reorganization is well established part of HF, and contributes importantly to disrupted cardiomyocyte function. (142) Furthermore, distribution as well as

organization of RyR2 clusters might be altered in HF, with potential implications for propagation of spontaneous Ca^{2+} release, (143) and efficiency of ECC. (144)

In sum, many well established and emerging perturbations in HF might contribute to an increased risk of SR Ca^{2+} leak and triggered activity.

4.6 Effects of exercise training on cardiomyocyte Ca^{2+} handling and triggered activity

This thesis explores exercise training (ET) as an antiarrhythmic strategy. In general, physical inactivity is a major health problem and the fourth leading cause of death worldwide. (145) Physical inactivity is estimated to cause 6 % of the burden of disease from coronary heart disease, and 9 % of premature mortality. (146) Today, the recommendations for primary prevention of cardiac disease comprise at least 150 min/week of moderate-intensity activity or 75 min/week of activity with vigorous intensity. (147) Even in older adults, it has been shown that ET can lower all-cause mortality. (148) However, only 29 % of men and 32 % of women meet this level of activity in Norway. (149)

ET is also an important part of secondary prevention for cardiac disease: Guidelines clearly states that all patients should be offered exercise as part of their rehabilitation program after a myocardial infarction. (150) More recently, the beneficial effects of ET in other cardiac conditions such as HF and atrial fibrillation have also been recognized, and included as part of efforts to modify risk factors for cardiovascular disease such as hyperlipidemia, obesity, hypertension and type 2 diabetes. (151-154)

The overall effects of ET on the cardiovascular system are well described: Long-term adaptations involve decreased afterload due to reduced peripheral resistance, increased maximal stroke volume due to increased ventricular volumes and contractile performance. (155) ET also increased maximal oxygen uptake (VO_{2max}) and aerobic interval ET has been shown to be superior to raise VO_{2max} levels compared moderate ET. (156) Importantly for this thesis, ET has been shown to reduce the propensity for VT and SCD after a myocardial infarction in animal models. (157, 158) These effects of ET are less established, and the potential mechanisms are the topic of this thesis.

Both clinical and translational studies indicate that the exercise intensity is important for the effects of ET. One study of patients with HF found that high-intensity ET improved aerobic capacity by 46 %, compared to 14 % after moderate-intensity ET, and was also associated with reversed left ventricle remodeling. (156) Others found that patients with left

ventricular hypertrophy and increased cardiac biomarkers had beneficial effect of high-intensity ET. (159) In this study, high-intensity ET also reversed left ventricular myocardial stiffness. (159)

Animal models of ET in rats showed that VO_{2max} levels increased more with high-intensity ET compared to moderate-intensity ET, and this increase correlated with alterations in cardiomyocyte function when fractional shortening, contraction, and relaxation rate was measured. (160) A study in healthy rats showed improved capillary density and cardiac hypertrophy after high-intensity ET. However, they found equal improvement in cardiac parameters such as EF, cardiac output and volumes with high and moderate-intensity ET. (160, 161) A mice model of with diastolic dysfunction because of obesity and prediabetes showed that high-intensity ET even can reduce the size of MI. (162)

Effects of ET on cardiomyocyte Ca^{2+} handling has been studied in a number of animal models, but is less established. A much cited study in rats showed that aerobic capacity increased with ET, but reached a plateau level after 6-8 weeks. (163) This study found an increase in ventricular weight after ET, increased myofilament Ca^{2+} sensitivity, and improved cardiomyocyte contractility already after 4 weeks of interval ET, while SERCA2 was upregulated after 13 weeks of ET. (163) Another potentially important observation after ET, was a down-regulation of NCX1 after ET in post-infarction dogs prone to ventricular fibrillation. This down-regulation coincided with decreased propensity for ventricular fibrillation. (164) Similar effects from ET on NCX1 were found in a dog model of pacing induced HF, where ET normalized the level of NCX1. (165) However, other results from rat models have shown ambiguous results of ET on NCX1. (163) One study of healthy rats who performed ET for 24 weeks, also found increased RyR2 abundance after ET. (166)

In one study of a rat model of HF after MI, ET normalized Ca^{2+} handling, (167) and other studies have found similar results. (168-170) In the context of this study, ET notably reduced spontaneous Ca^{2+} waves in a rat model of post-myocardial infarction HF. (171) This was supported by results from post-infarction mouse models. (172, 173) Beneficial effects of ET on SR Ca^{2+} leak has also been found in models of type 1 diabetes (174) and diabetic cardiomyopathy. (175) One proposed mechanism for these effects of ET were decreased activation of CaMKII. (172) Other studies suggest mechanisms that depend on reduced ROS production. (176) Campos *et al.* showed that ET restored dysfunctional mitochondrial ROS production in a rat model of heart failure. (177) Similar results have been found in a mice model of obesity, where ET prevented left ventricle dysfunction, improved mechanical

efficiency and decreased level of cardiac ROS after both moderate and high-intensity ET. (178)

5 Aims and objectives

The aim of this thesis was to gain improved understanding of the cellular mechanisms for triggered arrhythmias, and the effect of ET on these mechanisms.

The specific objectives of each subproject were:

Paper 1
To identify the individual and separate effects of heart rate and sympathetic activity on arrhythmia initiation in CPVT1.

Paper 2
To test the effect of ET on arrhythmogenic SR Ca^{2+} release in a mouse model of CPVT1.

Paper 3
To test the effect of ET on arrhythmogenic SR Ca^{2+} release in ventricular cardiomyocytes from a rat model of post-MI HF.

6 Materials and methods

Detailed descriptions of the material and methods used in this thesis is found in each paper in the appendix. In this section, general methodological considerations are discussed.

6.1 Patient population

For paper 1, we recruited patients with CPVT1 from the out-patient clinic at the Department of Cardiology at Oslo University Hospital Rikshospitalet, Oslo, Norway. All (sixteen) patients had CPVT1-associated *RyR2* mutations, and 11 (69 %) patients had experienced typical symptoms and/or ventricular arrhythmias during physical stress testing. All three patients included in the pacing protocol had experienced typical symptom with multiple syncopes. The patients included did not suffer from any other cardiac diseases, comorbidities, disabilities, or had planned major surgeries or medical treatments. None of the patients had the CPVT1-causativ *RyR2* R2474S missense mutation, which was the mutation of the transgenic mice used in paper 1 and 2.

6.2 Exercise training and cardiopulmonary exercise testing

There are two main options for exercise testing of patients: treadmill running and ergometer bicycling. We used bicycling for testing in paper 1. The reason is that this is the standard stress-test procedure for adults with CPVT1 at the Department of Cardiology at Oslo University Hospital Rikshospitalet. Benefits of bicycling stress-testing comprise more stable ECG recordings compared to ECG recorded during running. The use of ergometer bicycling in our study also enabled comparison to previous data, and ensured that the patients were already familiar with the protocol for testing. Tests were performed as a modified Bruce protocol for all patients, starting at a workload of 25 W, gradually increasing with 25 W every 2 minutes. (179, 180) If patients were not able to continue with the normal increase in workload, or the workload was too low to reach exhaustion, the interval between incremental workload was individualized. The participants reached exhaustion when they could no longer manage a cadence of 60 rpm. Patients with CPVT has been advised not to participate in sports because of the risk of arrhythmias, and many of the patients developed exhaustion in legs before reaching cardiac exhaustion and shortness of breath. The ECG recordings during testing allowed us to measure the heart rate before PVC in bigeminy occurred. This heart rate

threshold was set for each person, and was previously found to be reproducible for each individual. (2)

6.1 Electrophysiology in rodents and humans

For this thesis, we used mouse and rat models to understand human conditions. Importantly, however, extrapolation between mice and rats and humans should only be done with care and with several caveats in mind: (181) Most obviously, the resting heart rate in mice is 500-600 bpm and in rats 400-500bpm. (182) During exercise, heart rates reach app. 700-800 and 500-600 bpm, respectively. (182) The modest increase compared to humans reflects the high level of sympathetic activity at rest in rodents, however this elevated sympathetic activity could be due to experimental conditions such as temperature. (183)

The main ion currents involved in the normal action potential of the cardiac cycle involves multiple similarities in human and rats, and major aspects of mechanisms for triggered arrhythmias are the same. (184) However, some factors with importance for triggered arrhythmias should be noted when extrapolating results from rodents to humans. First, APs in rodents are very short compared to the humans: a typical ventricular AP in mice is 70-90 ms, compared to 400-500 in humans. (185) This is mainly due to a high expression of ion channels responsible for the transient outward potassium current (I_{to}) in phase 1 in rodents. There are also other species-dependent differences in the expression of potassium channels. The result is that the AP plateau in phase 2 is much longer in humans, and almost missing in mice. (186) Second, Ca^{2+} removal from the cytosol is more SERCA2-dependent in rats and mice (over 90%) than in humans (70%). Correspondingly, NCX accounts for only 7 % of cytosolic Ca^{2+} removal in rats, compared to 30 % in humans. (11) Of special importance for paper 1, the increase in SR Ca^{2+} content associated with increased stimulation frequency in human cardiomyocytes, is not found in mice. (187)

In paper 2, a subset of mice was used for ECG recording with telemetry. The mice were anesthetized with isoflurane 2 % inhalation, and buprenorphine 0.1 mg/kg was given subcutaneously in addition. We allowed a 7 day interval between surgery and ET to avoid any possible side effects of surgery or anesthetics.

The mouse ECG lacks a distinct T-wave and is characterized by a pronounced J-wave. (188) It was previously thought that the small cardiac mass makes mice less prone to develop ventricular arrhythmias. (189) However, later studies have shown that this is not in itself

prohibitive for ventricular arrhythmias and several mouse and rat models develop clearly recognizable sustained arrhythmias. (71, 73, 190)

6.2 Experiments with RyR2 R2474S mice

In paper 1 and 2, we used transgenic mice with the CPVT1-causativ *RyR2* R2474S missense mutation (RyR-RS). This transgenic mice model of CPVT1 has been used in other publications. (73, 191, 192) This mutation is also found in patients diagnosed with CPVT1. (73) Homozygous expression result in intrauterine lethality. The *RyR2* mutation leads to increased SR Ca^{2+} leak due to gain of RyR2 function. (73) The proposed mechanism for this is disruption of FKBP12.6 binding to RyR2, which results in increased RyR2 activity. (73) The mice exhibit arrhythmias similar to those observed in patients with CPVT1, i.e. the pathognomonic bidirectional ventricular tachycardia. (73) However, since the mechanism underlying this special type of arrhythmia is not entirely clear, we cannot definitely say that it reflects the same electrophysiological process. (73) RyR2 R2474S mice also exhibit epileptic seizures, (73) although in our lab such events have never been witnessed.

6.3 Rat model of heart failure

In paper 3 we used a rat model of HF, i.e. Wistar rats subjected to left coronary artery ligation for induction of myocardial infarction and HF development. This model has been used by many research groups, and in a number of projects in our institute. (193-195) Our surgical procedure for coronary artery ligation is well established, even with regard to expected size of MI and HF phenotype from a specific position of the ligation, and the post-operative follow-up and expected development is well known. Compared to mice, the yield from cell isolation is greater with rats, and rat cardiomyocytes are generally more stable. Each rat heart also provides tissue for other experiments. These factors are important when choosing a model for the time and resource demanding ET protocols.

We studied rats to understand human HF. Although our model has many similarities with post-infarction HF in humans, it also has some obvious and notable differences. First, HF in these rats is not multifactorial, as opposed to human HF in which hypertension, diabetes, obesity, systemic atherosclerosis and other comorbidities often contribute in synergy. (196, 197) However, the reductionist and highly standardized approach offered by our model is also the strength when studying mechanisms in post-infarction HF, and enables comparison to

previous studies. Nevertheless, notable differences to other models in terms of species, strains and specifics of the protocol must still be acknowledged when comparisons or made. Second, the observation period after infarction and time for ET is relatively short. However, HF in humans also develop shortly after MI, and remodelling and scar tissue development starts immediately. At six weeks, the scar is relatively stable even in humans. (198) Furthermore, patients should start rehabilitation programs in-hospital after MI, and continue one to three weeks after hospital discharge. (199) Thus, the timing of our protocol is relevant for patients, although many processes in rodents occur at a much higher rate and more rapidly than in humans.

6.4 Measurements of aerobic capacity and effects of exercise in rats and mice

In paper 2 and 3 we investigated whether the propensity for spontaneous SR Ca^{2+} release can be altered by ET. ET was performed as treadmill running with a separate treadmill and metabolic chamber used for weekly measurements of maximal oxygen uptake (VO_{2max}, ml/kg/min). This method is reliable in both mice (200) and rats. (201) The adaptation after ET is similar in mice, rats and humans even if the heart rate in rats and mice only increase by around 40 % during exercise. (182, 200, 201) The exercise training protocol was as follows: Three days before the first VO_{2max} test, rats and mice were habituated to treadmill running with daily 15 min runs on the treadmill at walking pace. Both rats and mice adapted to treadmill running after the initial 3 days, and were able to perform the running test for maximal oxygen uptake at week 0 of the ET protocol.

Both mice and rats were randomized to either ET or SED protocol. High-intensity ET has been shown to increase aerobic capacity in rats more than moderate ET (160). The running speed was adjusted for all animals based on weekly measurements of VO_{2max} to keep the intensity at high-intensity throughout the program. The VO_{2max} test was performed in a closed chamber made especially for either rat or mouse. We recorded six VO_2 measurements at each speed, and the average of the last 3 measures at the highest performed speed was set as VO_{2max}. Exhaustion was reached when five electric stimuli was given within 15 s for both mice and rats. Three investigators performed the ET part of the studies in paper 2 and 3. To reduce variation in test-conditions, each cohort of mice and rats was tested by the same investigator, if possible. Although bouts of ET were supervised by an individual investigator, some of the bouts and the VO_{2max} tests were done jointly by all investigators to standardize the procedure and reduce variation between personnel.

After initial testing, the mice completed an ET protocol of two weeks, and the rats completed five weeks of ET. The ET protocol for mice was set to two weeks after performing a pilot ET protocol with WT mice. The five week ET protocol for rats was based on previous studies showing that VO_{2max} reaches a maximum value after 5 weeks of ET. (160) In our experience, mice seem more eager to run on the treadmill, however both mice and rats were able to perform the interval ET program. Treadmill running allowed us to control the running intensity more closely compared to other available ET modalities, such as swimming or voluntarily wheel running. The rodents are exposed to a minor risk of injuries to feet and tail during treadmill ET, and therefore needed to be monitored by an investigator at all time during an ET-session. However, with adaptation to the ET protocol and surveillance by a researcher during ET, we did not experience injuries to either rat or mice performing ET. Both mice and rats also quickly learn to avoid the electric grid located at the end of the treadmill, however this could potentially add extra stress to the ET protocol.

6.5 Mouse and rat echocardiography

Echocardiographic parameters (Vevo2100 System, VisualSonics Inc., Canada) were recorded during general anesthesia before and after completion of the ET program. (202) Because of the high heart rate and restricted "acoustic window", it is technically difficult to perform imaging in rodents, and operator dependence and experience is of great importance. All mouse and rat echocardiographic exams for the different papers were performed by the same person, and conditions were standardized as much as possible by controlling the level of anesthesia based on heart rate, temperature of the animals with a heat mat, and handling of animals prior to the procedure. Isoflurane has a cardio-depressive effect, but less than other anesthetics that can be used for research animals. (203, 204) The anesthesia was kept as light as possible monitored by HR, but nevertheless, we did not perform VO_{2max} test on the same day as echocardiography. When analyzing the images obtained from echocardiography, the investigators were blinded for group affiliation.

6.6 Isolation of ventricular cardiomyocytes

Ventricular cardiomyocytes were isolated from mice and rats using constant pressure perfusion of the coronary arteries with collagenase solution containing low Ca^{2+}, as previously described. (205) Some challenges associated with isolation of adult

cardiomyocytes should be noted: Collagenase used for dissolving the extracellular matrix comes with varying enzymatic activities, and needs to be tested for each batch. This means that different concentrations of collagenase are needed for each batch, which leads to somewhat unpredictable results in terms of isolation quality. Also, the quality of the cells used for experiments is assessed by eye, which is a source of variation among different researchers. We tried to overcome these problems by only using clearly striated cardiomyocytes with visually intact cell membranes, without spontaneous contraction for 10 s in a physiological solution containing 1 mM Ca^{2+}. Furthermore, we aimed to have one investigator performing each experimental series, in order to standardize conditions as much as possible. We aimed to perform multiple types of experiments with cells from each cell-isolation, both to make results as comparable as possible, and to reduce the number of animals needed in total. For cellular experiments involving ET and SED animals, cardiomyocytes from both groups were isolated at the same time and the isolation was performed by an investigator blinded to the experimental group.

6.7 Cellular and subcellular Ca^{2+} imaging

In vitro experiments for paper 1, 2, and 3 were performed with whole-cell Ca^{2+} imaging and confocal microscopy. The benefit of using isolated cardiomyocytes is that conditions can be highly controlled and standardized. Nevertheless, isolated cardiomyocytes lack the influence of the neighboring cells in the intact heart, as well as neurohormonal effects. Although key aspects of Ca^{2+} handling in isolated cardiomyocytes is very similar to Ca^{2+} handling in cardiomyocytes studied *in situ* in intact hearts, (206, 207) the potential differences somewhat limits the extrapolation of results from cardiomyocytes to the intact heart.

Cytosolic Ca^{2+} was visualized with fluo-4AM (Molecular Probes, Eugene, Oregon, USA), after loading of cardiomyocytes for 10 min prior to experiments. All experiments were performed at 37°C. Isoprenaline (ISO) 100 nM (NAF, Norway) was used to stimulate beta-adrenoceptors. A stable beta-adrenoceptor stimulation response was ensured by starting the experimental protocol 60 sec after the start of ISO-exposure. The response was validated by a visual increase in Ca^{2+} transient amplitude, based on previous experiments with the same model in our lab. For these experiments, the concentration of ISO in the experimental chamber would not depend on flow rate. However, for experiments with rapid exposure to high-dose caffeine, flow-rate would be important. We therefore standardized the distance

from the outflow of the perfusion system to the visual field by visual inspection in the microscope, and standardized the flow-rate in the system. To avoid effects of previous stimulation and time-dependent effects, we varied the order of stimulation frequencies when several stimulation frequencies were applied to the same cell. Ca^{2+} sparks was recorded by confocal microscopy line scan imaging, with the same considerations before performing the experiments when it comes to cardiomyocytes in normal conditions after isolation and for beta-adrenergic response. Confocal line scan imaging is restricted to a small area of the cardiomyocyte. It is also limited by the preset timing for scanning in a set timeframe, which could potentially leave out Ca^{2+} sparks from other parts of the cardiomyocyte and in a different time-window after the last Ca^{2+} transient.

6.8 Quantification of cardiac proteins

Western blots were performed on total protein homogenates from mouse and rat left ventricles stored at -70°**.** For paper 1 the hearts were paced at 4 and 8 Hz before the tissue was rapidly frozen. Simultaneous recording of ECG was performed to document the pacing frequency. After pilot experiments, we were able to pace the hearts at a stable frequency for 3 min. The hearts were kept stable by perfusion through the aorta on a modified Langendorff setup. Importantly, rapid freezing in liquid nitrogen was secured at the immediate end of the protocol to keep posttranslational modifications, including phosphorylation, as stable as possible. (208) Western blots of Ox-CaMKII performed for paper 2 were especially challenging: To avoid reduction of the oxidized protein a special non-reducing buffer had to be used. Two different antibodies were used, one custom-made for our lab, and one acquired from our collaborators in Professor Mark Anderson`s group (Johns Hopkins Hospital, Baltimore, MD, USA). Blots made with these antibodies showed similar results.

The semi-quantitative nature of Western blotting makes interpretations of small differences difficult. In paper 2, we found a small, but statistically significant difference in the abundance of Ox-CaMKII between ET an SED groups. Partly due to the small numeric difference between groups, we validated the results in repeat experiments. The interpretation of these results are further discussed in the Discussion.

For paper 3, we quantified beta-adrenoceptor density by radioligand binding essays. These experiments were performed with tissue from rat left ventricles snap frozen in liquid nitrogen and stored at -70°. These experiments determined membrane bound density of both the $beta_1$-adrenocepotor density and the $beta_2$-adrenoxceptor density, compared to the total

abundance of the receptors measured by Western blotting. We used the selective ligand CGP20712A for $beta_1$-adrenoceptor measurements, and the selective ligand ICI118551 for $beta_2$-adrenoceptor measurements. The radioligand [^{125}I]-(–) iodocyanopindolol is a common used radioligand for labeling and measure $beta_1$-adrenoceptor and $beta_2$-adrenoceptor density, and has been shown to have high affinity and specificity to beta-adrenoceptors. (209)

6.9 Simulation of Ca^{2+} handling in CPVT cardiomyocytes by computer modeling

A computer model of Ca^{2+} handling was used in paper 1 to dissect and separate the effects of beta-adrenoceptor stimulation and stimulation frequency on Ca^{2+} wave development, and to elucidate the underlying mechanisms. The computer model was based on an existing model of Ca^{2+} handling in cardiomyocytes. (134) These experiments were performed by interaction between cellular and computational experiments: Data from cellular experiments were used to set the essential parameters in the model in order to recreate key elements of Ca^{2+} handling in WT and CPVT mouse cardiomyocytes. The next step was to perform simulations of the cellular experiments, and to isolate the contribution of individual factors. These experiments offered detailed possible explanations for the results observed during cellular experiments. The obvious caveat is that modeling experiments are limited by the input to the model and simplified versions of the highly complex system present in cardiomyocytes.

6.10 Statistics and experimental design

For all cellular experiments, the investigators were blinded for group affiliation to ET or SED. This was ensured by collection of animals by others than the investigators performing cellular experiments. We used cells from each cell isolation to different cellular experiments to reduce the number of animals used, and to ensure most efficient use of the cardiomyocytes harvested from the cell isolations. When measuring Ca^{2+} sparks, we counted Ca^{2+} sparks in a set timeframe (in a 6 s post-stimulation rest period) after the last stimulated Ca^{2+} transient. Many of the cells had zero Ca^{2+} sparks, and to control for possible skewed distribution and no normal distribution, a Poisson test was performed. In paper 2 and 3 a paired Student's *t*-test was used to compare the effect of exercise training on VO_{2max} at baseline and at the end of ET period. Unpaired nested ANOVA was used to compare groups for all results from whole-cell

Ca^{2+} imaging. In addition, two-way ANOVA was used to compare difference between ET and SED in the presence or absence of ISO at all frequencies for Ca^{2+} waves, Ca^{2+} transient amplitude, and Ca^{2+} decay rate in paper 3.

7 Summary of the main results in the thesis

This is a summary of the main results in each of the original papers included in this thesis. For full results, please see the full manuscript of each paper in the appendix.

7.1 Paper 1: Arrhythmia initiation in catecholaminergic polymorphic ventricular tachycardia type 1 depends on both heart rate and sympathetic stimulation

In paper 1 we aimed to untangle and study the separate effects of HR and beta-adrenoceptor stimulation on arrhythmia initiation in CPVT1. Sixteen patients with CPVT1 performed a bicycle stress-test with continuous ECG recording until exhaustion. ECGs recorded during the stress-test were used to identify an individual threshold for first occurrence of PVCs. Three patients with permanent two chamber ICDs volunteered to atrial pacing above the individual threshold for PVCs identified during the bicycle test in the same consultation. In none of these patients did atrial pacing elicit PVCs.

The basic mechanisms for the clinical observations were explored by experiments with isolated ventricular cardiomyocytes from RyR-RS mice. Increasing pacing frequency increased the number of Ca^{2+} waves in both RyR-RS and WT cardiomyocytes in absence of beta-adrenoceptor stimulation. However, during exposure to ISO, the frequency of Ca^{2+} waves increased more in RyR-RS than in WT cardiomyocytes.

A computer model of CPVT1 which reproduced the cellular CPVT1 data, showed that pacing frequency and beta-adrenoceptor stimulation both contributed to initiation of Ca^{2+} waves in a mechanism that involved CaMKII-dependent RyR2 phosphorylation and timing of SR Ca^{2+} reloading.

This study confirmed the heart rate-independent pro-arrhythmic effect of beta-adrenoceptor stimulation in CPVT1, but also identified an independent and synergistic contribution from high heart rate.

7.2 Paper 2: Exercise training prevents ventricular tachycardia in CPVT1 due to reduced CaMKII-dependent arrhythmogenic Ca^{2+} release

In paper 2, we aimed to test the effects of ET on arrhythmogenic Ca^{2+} release events in RyR-RS mice. RyR-RS mice were randomized to two weeks of ET or SED. ET increased VO_{2max} by 10±2 %, while no change was observed in SED controls. RyR-RS ET mice (RyR-

RS ET) had fewer VT episodes than RyR-RS SED immediately after a stress test. Correspondingly, cardiomyocytes from RyR-RS ET showed fewer Ca^{2+} sparks and Ca^{2+} waves, and less diastolic SR Ca^{2+} leak. Protein analysis showed lower levels of CaMKII-dependent phosphorylation of RyR2 in RyR-RS ET, and reduced abundance of oxidized CaMKII. This coincided with a reduction of Ca^{2+} waves in RyR-RS cardiomyocytes to ET levels after inhibition of CaMKII with autocamtide-2-related inhibitory peptide (AIP) or the antioxidant N-acetyl-I-cysteine (NAC).

We concluded that long-term effects of interval treadmill ET reduce Ca^{2+} release events underlying triggered activity and VT in mice with a CPVT1-causative *Ryr2* mutation through lower CaMKII-dependent phosphorylation of RyR2.

7.3 Pape 3: Exercise training stabilizes RyR2-dependent Ca^{2+} release in post-infarction heart failure.

In paper 3, we investigated if ET had the potential to stabilize RyR2-dependent Ca^{2+} release in rats with post-MI HF. Male Wistar rats were subjected to left coronary artery ligation or sham operations. One week after surgery, animals were characterized by echocardiography and randomized to high-intensity interval ET (HF-ET) on treadmills or to SED (HF-SED). Running speed was adjusted based on a weekly VO_{2max} test. We repeated echocardiography after 5 weeks of ET and harvested left ventricular cardiomyocytes for analysis of RyR2-dependent systolic and spontaneous diastolic Ca^{2+} release.

ET increased VO_{2max} in HF-ET rats to 127% of HF-SED. Ventricular diameter and fractional shortening were unaffected by ET. Left ventricular cardiomyocytes from HF-ET exhibited attenuated spontaneous SR Ca^{2+} release, but also reduced Ca^{2+} transient amplitude and slowed Ca^{2+} reuptake during beta-adrenoceptor stimulation. Analysis of Ca^{2+} homeostasis and major proteins involved in the regulation of SR Ca^{2+} release and reuptake could not explain the attenuated spontaneous SR Ca^{2+} release or reduced Ca^{2+} transient amplitude. Interestingly, measurements of beta-adrenoceptors showed a normalization of $beta_1$-adrenoceptor density and $beta_1$:$beta_2$-adrenoceptor ratio in HF-ET.

We concluded that ET increased aerobic capacity in post-MI HF rats and stabilized RyR2-dependent Ca^{2+} release. Our data showed that these effects of ET can be gained without major alterations in SR Ca^{2+} regulatory proteins, and indicated that future studies should include upstream parts of the sympathetic signaling pathway.

8 Discussion

8.1 Mechanisms for arrhythmias in CPVT – new insights in RyR2-dependent triggered activity

It is well established that VT in CPVT is caused by triggered activity due to increased RyR2-dependent diastolic SR Ca^{2+} release that elicit DADs. (24, 71-73) It is also well established that almost, if not all, CPVT-associated mutations known to date, affects RyR2 function directly or through RyR2-modulators. Therefore, CPVT has gained a lot of attention as a "model disease" for triggered activity. However, studies such as the ones presented in this thesis, illustrate the need for separate studies of mutations that affects RyR2 differently on a molecular level, even if their cellular and *in vivo* phenotypes have many similarities. Additionally, a detailed understanding of mechanisms for triggered arrhythmias in other conditions such as HF are needed before extrapolations can be made from monogenic diseases such as CPVT.

Indeed, the literature shows that the mechanisms resulting in diastolic Ca^{2+} release might vary between different mutations and conditions. One way to categorize these differences and their functional implications, is to start with the fact that beta-adrenoceptor stimulation can both increase SR Ca^{2+} concentration and the Ca^{2+} sensitivity of the RyR2, i.e. lower the threshold for SR Ca^{2+} release. Which of these factors that dominate the phenotype varies between different mutations and is still not clearly described many mutations. E.g., even if all of the mutations *RyR2* R2474S, (73) *RYR2* R4496C, (71) and *RyR2* R176Q (210) result in a seemingly similar CPVT phenotype we do not know if their responses to beta-adrenoceptor are due to the same underlying effect on release threshold and SR Ca^{2+} reuptake. Cellular experiments from RyR2 R4496C mice showed an increased Ca^{2+} sensitivity of the RyR2 upon beta-adrenoceptor stimulation, (211) but other experiments showed that beta-adrenoceptor stimulation did not change the threshold for diastolic SR Ca^{2+} release, but rather increase SR Ca^{2+} concentration above the threshold. (212) We do not know if the same is true for the other RyR2 mutations, or if the dominant effect of beta-adrenoceptor stimulation in those mutations might be to lower the threshold for release even further.

Another way to illustrate the need for detailed and separate studies of different mutations and conditions is the complexity in interpretation of acute responses to exercise. It is well established that a bout of exercise increases sympathetic activity and decreases vagal tone, leading to increased heart rate, conduction velocity in the myocardium, and increased

cardiac inotropy. (213-215) However, a number of signaling pathways of potential importance for triggered activity are activated in this process: PKA activation is increased due to increased cAMP production; (216) ROS production from both cytosolic and mitochondrial sources is also increased; (217) and CaMKII is activated by a number of pathways, including EPAC, ROS and cytosolic Ca^{2+}. (218, 219) (123, 220) Effects of ET are therefore not easily interpreted, as are effects of therapy since beta-blockers (mostly) act upstream of the different signaling mechanisms and the mechanism for the effects of flecainide is still a matter for debate. (96)

Even if the downstream effects of ET are complex, CaMKII seems to be an important mediator of beta-adrenoceptor stimulation in CPVT. As en example, Liu *at al.* showed in a mouse model of CPVT (R4496C), that inhibition of CaMKII reduced Ca^{2+} spark frequency and fractional release of Ca^{2+} from the SR. This effect of CaMKII inhibition was most present during beta-adrenoceptor stimulation by ISO. (220) Berisha *et al.* has shown that beta-adrenoceptor stimulation phosphorylate RyR2 by increased cAMP levels. They further showed that this led SR Ca^{2+} leak and possibly arrhythmia susceptibility in both failing mouse and human cardiomyocytes. (221) It has been shown by others that SR Ca^{2+} leak is increased during beta-adrenoceptor stimulation activating by CaMKII-dependent phosphorylation of RyR2, and inhibition of CaMKII restored the SR Ca^{2+} leak to normal levels. (123) In a gene modified mouse model of CPVT (RyR2-R176Q), inhibition of CaMKII suppressed ventricular arrhythmias and restored abnormal Ca^{2+} handling. (222) The same study showed that CaMKII inhibition in human stem cell derived cardiomyocytes with CPVT, from two different pathogenic mutation, normalized Ca^{2+} handling. (222)

In paper 1 we wanted to further understand the effects of acute exercise on arrhythmias in CPVT by starting with a basic observation, i.e. that arrhythmias in CPVT occur with increasing frequency and severity with increasing heart rate. Specifically, we wanted to separate the effects of increased heart rate *per se* from other effects of beta-adrenoceptor stimulation. This separation is not achievable *in vivo*, and we therefore combined patient data, cellular data from a mouse model of CPVT, and a computer model to elucidate the mechanism. From this, we confirmed that beta-adrenoceptor stimulation is a necessary factor to reveal the increased propensity for arrhythmogenic SR Ca^{2+} release events, but also showed that increased frequency contributes to arrhythmogenesis.

Although the focus in previous literature on CPVT is mostly focused on effects of beta-adrenoceptor stimulation in cardiomyocytes, our results in paper 1 are supported by previous studies: Endocardial monophasic action potentials showed DADs even in the

absence of beta-adrenoceptor stimulation, but with an increased frequency in presence of beta-adrenoceptor stimulation, in patients with CPVT performing an invasive electrophysiological examination. (223) Furthermore, in mice with a *RYR2* (R4496C) mutation that cause CPVT, it has been shown that frequency increases the propensity for Ca^{2+} waves and DADs even without beta-adrenoceptor stimulation present. Similar to our results, this study found an additive effect of beta-adrenoceptor stimulation on the frequency of Ca^{2+} waves in CPVT cardiomyocytes. (211)

The interpretation of the results in paper 1 in terms of mechanisms for arrhythmias in CPVT1 are not straight forward: We focused on CaMKII based on the assumption that CaMKII is sensitive to increased heart rate. This has been shown in a computer model based on Ca^{2+} handling from rabbit cardiomyocytes, where increased pacing frequency increased CaMKII activity, with subsequent phosphorylation of RyR2. In this model, increasing pacing frequency from 2 Hz to 3 Hz increased SR Ca^{2+} leak and triggered DADs even in absence of ISO during CaMKII-overexpression. (224) Our results in paper 1, suggest that the beta-adrenoceptor stimulation leads to both faster Ca^{2+} refilling of the SR and lower SR Ca^{2+} threshold for SR Ca^{2+} release, and that CaMKII contributes to both of these effects.

One unresolved aspect is whether the threshold for SR Ca^{2+} release is lower than WT even at baseline, or whether this is a result of the effects of beta-adrenoceptor stimulation and/or increased frequency. We also found lower Ca^{2+} transients and lower SR Ca^{2+} threshold for Ca^{2+} waves in RyR-RS cardiomyocytes compared to WT cardiomyocytes during beta-adrenoceptor stimulation, which could be due to increased SR Ca^{2+} release that is not compensated for by sufficient Ca^{2+} filling of the SR. Similar results have been found in cardiomyocytes with the *RyR2* R4496C mutation. (212) As already described, the results from the R4496C mice were interpreted as the main effect of beta-adrenoceptor stimulation was to increase the SR Ca^{2+} concentration, and not to change the SR Ca^{2+} threshold. (212) However, Uchinoumi *et al.* proposed that the SR Ca^{2+} threshold was lower in CPVT cardiomyocytes compared to WT cardiomyocytes due to changes in the interdomain interaction of RYR2, and more sensitive to PKA-dependent phosphorylation pf RyR2 during beta-adrenoceptor stimulation. (75) Importantly, there was no difference in the phosphorylation level of RyR2 Ser2808 between CPVT cardiomyocytes and WT cardiomyocytes.

In summary, our results reveal the complexity in the details of mechanisms responsible for triggered arrhythmias even in a monogenic disease such as CPVT. Even though we confirm the consensus view that beta-adrenoceptor stimulation is a necessary factor in the development of arrhythmias in CPVT, our findings which indicate an independent role for

increased heart rate, illustrate the need for further elucidations of the mechanisms involved, and for studies of individual mutations and conditions.

8.2 Effects of exercise training on Ca^{2+} dependent arrhythmias in CPVT

In the studies included in this thesis, we have studied both the acute effects of exercise and the long-term effects of repeated exercise, i.e. ET. Based on previous observations of ET-effects on Ca^{2+} handling, we hypothesized that some of these effects would also be beneficial in CPVT. In paper 2 we found that ET reduced SR Ca^{2+} leak and ventricular arrhythmias in the RyR2 R2474S mice, with supportive indications from a pilot study of patients with CPVT from our group. (225) The findings of reduced diastolic SR Ca^{2+} release in paper 2 are in line with previous studies on effect of ET in other disease models with increased SR Ca^{2+} release. (157, 171, 175)

Several mechanisms could contribute to these effects of ET in CPVT. One explanation could be upstream effects on the activity in the autonomic nervous system. Patients suffering from MI, showed improved parasympathetic function after ET, (226) while in a dog model of MI, ET restored beta-adrenoceptor balance, and prevented VF. (227) In paper 2, we only studied potential downstream effects of beta-adrenoceptor stimulation and found a reduction in CaMKII-dependent phosphorylation of RYR2. We chose to test the specific hypothesis that reduced oxidation could play a role for this effect. This was based on previous studies showing increased antioxidative capacity in the heart after ET, (177) and the fact that beta-adrenoceptor stimulation which is essential for arrhythmia development in CPVT increases the level of ROS. (228) We found that Ox-CaMKII was reduced in ET mice, and that this coincided with reduced CaMKII-dependent phosphorylation of RyR2 together, reduced SR Ca^{2+} leak and lower incidence of ventricular arrhythmias in vivo after ET. This effect of ET was present together with lower levels of oxidative stress, measured as malondialdehyde levels. Subsequent studies by others have shown that mitochondrial function is altered in CPVT, and increased levels of ROS derived from mitochondria has been measured. (229) This might also have contributed to our results. Furthermore, this mechanism of increased SR Ca^{2+} leak could also be relevant for HF, where increased mitochondrial ROS production plays an important role in many models. (230)

Modulation of CaMKII after ET could also be due to other mechanisms. Qin *et al.*, found that ET reduced CaMKII and CaMKII-phosphorylated RyR2 after ET in a mouse model of post-infarction HF, and proposed that this lead to lower propensity for ventricular

arrhythmias due to restored autonomic balance and Ca^{2+} handling. (173) Interestingly, this study also found that the effect of ET was similar to the effect of treatment with bisoprolol. In view of these potential different mechanisms for reduced CaMKII activity, the beta-adrenoceptor antagonist carvedilol is especially interesting, as it also has a prominent antioxidant effect, and has been shown to reduce ROS and improve Ca^{2+} handling in HF cardiomyocytes from dogs. (231) No systematic studies of the effects of carvedilol has been performed in patients with CPVT, and the effect of carvedilol could not be assessed in a newly published multicenter study, due to a small number of patients with CPVT using carvedilol. (91)

Importantly for the interpretation of the results in paper 2, is that our group has tested the effect of reduced oxidation of CaMKII in the RyR-RS mice further in a follow-up study. (232). In this study, the RyR-RS mice were crossed with mice carrying CaMKII resistant to oxidation of methionines 286/287. However, this mutation did not prevent arrhythmogenic Ca^{2+} waves. Interestingly, genetic ablation of oxidized CaMKII did not alter RyR2 Ser2814 phosphorylation. The effect of ET found in paper 2 is therefore probably not the explained by effects on oxidized CaMKII, at least not completely. Alternative hypotheses could be reduced direct oxidation of RyR2, (233) or involve protein phosphatase 2A which dephosphorylates RyR2 Ser2814, (234) and has been shown to be upregulated after ET. (173) Finally, as specified above, paper 1 did not explore the upstream effects on the activity in the autonomic nervous system, and our results from effects of ET in HF rats in paper 3 could suggest an important role for such effects.

In summary, CaMKII seems important in CPVT, and a potential target for therapy, although the optimal way to achieve CaMKII inhibition remains elusive. Also important, the beneficial effects of reduced CaMKII abundance and/or activity is not dependent on increased CaMKII at baseline or altered effects of CaMKII on RyR2 in CPVT. The sum of proarrhythmic factors would nevertheless be reduced by reducing CaMKII activation.

8.3 Effects of exercise training on Ca^{2+} dependent SR Ca^{2+} release in heart failure

Although alterations of Ca^{2+} handling in HF are much more complex than in CPVT, as discussed above, the mechanisms for triggered arrhythmias due to RyR2 dysfunction have similarities. Beta-adrenoceptor stimulation is increased in HF, as are increased ROS levels. These alterations in HF could promote activation of CaMKII and downstream of CaMKII, activation of RyR2 and increased propensity for SR Ca^{2+} leak trough RyR2 and increased risk

of arrhythmias during HF. (112) Patients with HF also often have diabetes, which has been shown to contribute to increased CaMKII activation in a mice model of diabetes. (175)

In paper 3 we studied the antiarrhythmic potential for ET in HF, and if ET could modulate phosphorylation levels of RyR2. Similar to paper 2, we found seemingly beneficial effects on diastolic Ca^{2+} release in HF, with reduced SR Ca^{2+} leak measured as Ca^{2+} sparks and Ca^{2+} waves. This is in line with findings in a different rat strain with HF. (171) We found indications of altered beta-adrenoceptor composition in the ET group, but no alterations in phosphorylation of RyR2. One could speculate that the Ser2808 and Ser2814 phosphorylation sites are not the most important for the effect of ET, as the RyR2 S2030 site has been shown be some to play an important role in the response to beta-adrenoceptor stimulation in both healthy and HF hearts. (235) However, others have found reduced CaMKII phosphorylation of RyR2 Ser2814 after ET, with corresponding reduction in Ca^{2+} spark frequency and lower *in vivo* propensity for VT in a canine model of anterior MI. (157)

The role of CaMKII activation and phosphorylation of RyR2 Ser2814 might also depend on the etiology for HF: In a mouse model of HF after transverse aortic constriction, SR Ca^{2+} release and HF development corresponded to increased CaMKII activity and increased phosphorylation of RyR2 Ser2814, but not in HF after MI. (127) Paper 3 showed that other mechanism than RyR2 phosphorylation could also play a contributing role in stabilizing RyR2 and lowering the SR Ca^{2+} leak. In view of our results in paper 3, as well as the lessons from our CPVT mouse model, the mechanisms for ET-dependent reduction of diastolic Ca^{2+} release, is likely multifactorial.

8.4 Lessons from CPVT and heart failure - relevance for other conditions

Keeping the lessons on complexity and need for specific studies of different conditions discussed above in mind, RyR2 dysfunction is also part of the pathophysiology in other heart diseases, and in the findings in paper 2 and 3 might therefore have a wider relevance. The combination of increased ROS levels and activation of CaMKII are also present in other conditions, and modulation of ROS, CaMKII and thereby RyR2 function has showed promising results: Liu *et al.* showed that mitochondria from pluripotent stem cell-derived cardiomyocytes from patients with Barth syndrome had higher ROS production, increased activation of CaMKII and increased SR Ca^{2+} leak than controls. (236) Inhibition of ROS and CaMKII in these cells caused lower RyR2 phosphorylation at Ser2814 and lower SR Ca^{2+} leak. Similar results have been shown in a mouse model of Duchenne muscular

dystrophy, with normalization of Ca^{2+} handling and lower incidence of VT after reduced levels of oxidized CaMKII. (237)

CaMKII activity has also been found to be increased in diabetic cardiomyopathy, and linked to increased ROS production. (238) Even in a mouse model diabetic cardiomyopathy, ET lowered SR Ca^{2+} leak by reducing CaMKII activity. (175) Atrial fibrillation has also been linked to ROS and activation of CaMKII by increased oxidation. (239) Oxidized CaMKII has been found at elevated levels in atria from patients suffering with atrial fibrillation compared to patients with sinus rhythm, (239) and a mouse model of atrial fibrillation showed that reduction in oxidized CaMKII reduced the burden of atrial fibrillation. (239) Increased oxidation of RyR2 together with increased SR Ca^{2+} leak has also been found to be present in atria from patients with atrial fibrillation. (240) Pharmacological treatment of RyR2 dependent SR Ca^{2+} leak and genetic inhibition of ROS levels prevented atrial fibrillation. (240) In another model of atrial fibrillation involving the RyR2 mutation RyR2-R2474S, reduced oxidation stabilized RyR2 through restored FKBP12.6 binding, thereby reducing the AF burden. (241)

The results from these different conditions, shows that there are promising and possibly anti-arrhythmic effect of lowering ROS levels and CaMKII activity. However, the activation of CaMKII is multifactorial and complex, and inhibition of one activating factor can therefore be compensated by other activating factors. ET could have the potential to reduce the activation of CaMKII and alter the activity of RyR2 to a more stable state with lower SR Ca^{2+} leak through multiple targets in the beta-adrenoceptor signalling pathways and regulating Ca^{2+} handling in cardiomyocytes.

8.5 Clinical perspective

ET is an important part of both primary and secondary prevention of cardiovascular disease. (147) Even if ET is widely recommended for healthy persons and patients with cardiac conditions, some patient groups are advised to avoid ET, or only participate in sports after a throughout risk evaluation and shared decision making. This applies to patients with arrhythmogenic cardiomyopathy, inherited ion channelopathies, and dilated cardiomyopathy, especially due to lamin AC mutations. (147) For patients with CPVT, Ackerman *et al.* has proposed the following precaution before participating in sports: "*For an athlete with previously symptomatic CPVT or an asymptomatic CPVT athlete with exercise-induced premature ventricular contractions in bigeminy, couplets, or nonsustained ventricular*

tachycardia, participation in competitive sports is not recommended except for class IA sports (Class III; Level of Evidence C). Exceptions to this limitation should be made only after consultation with a CPVT specialist." (242) Nevertheless, studies have showed that around 50 % of persons with a heart condition recommended not to do exercise training still participate in these type of activities, against guideline recommendations. (243, 244)

High intensity ET has been shown to be safe even in patients with HF, and to result in increased aerobic capacity superior to moderate ET. (156) Our group has been the first to perform a study to investigate the effect of ET in patients with CPVT1. Our results show that it is possible for patients with CPVT to perform ET at moderate to high moderate intensity. The patients performed a total of 168 sessions of ET, without any adverse events. (225) The same study showed that the threshold for arrhythmias was increased after the ET period, and that the effect was lost upon cessation of ET. (225) Together with our experimental data from the mouse model in paper 2, this supports a real antiarrhythmic effect from ET for patients with CPVT.

Similar results have been found by others, with no increased risk of cardiac events in patients with CPVT participating in sports. Ostby *at al.* also showed that after been well informed, as many as 87 % continued sports after being diagnosed with CPVT. (243) Patients with long QT syndrome also continue with sports after being diagnosed, but with a lower overall rate of cardiac events. (245) The reasons why some patients choose to stop ET after being diagnosed with CPVT while others do not, is not clear. It is known that the phenotype of CPVT is different due to different mutations sites and specific genotypes. (60) On could speculate that with a more severe phenotype, and the experience of a serious cardiac event by index patient or close relatives, influence this decision. However, many patients with CPVT continue with exercise as a leisure time activity. Patients with CPVT participate in sports, (243) and the results from Manotheepan *et al., (225)* together with the results from paper 2, showed possible beneficial effects of ET on the propensity for arrhythmias for patients with CPVT. In addition to this, ET obviously has other health benefits associated with an active lifestyle. Of course, the level of exercise has to be individualized and reviewed at regular ambulatory visits. It is also important that patients are well medicated before participating in ET. Studies like the ones presented in this thesis, and the follow-up studies from our group will help clinicians to advice these patients. This type of research is especially important in rare disease where large randomized clinical trials are not feasible. Importantly, our results indicate that ET at a moderate and individual intensity level could be beneficial for patients with CPVT, (225) this is still not recommended in guidelines, and beta-blockers are the first

line therapy for CPVT. (61) Furthermore, even though beta-blockers with an antioxidant effect are an interesting concept in view of our results, and carvedilol has been shown to have this type of effect, (246) nadolol is the drug of choice in these patients according to current clinical evidence. (91) Other interesting options in light of our results include ivabradine and dantrolene. (247, 248) The proposed mechanism for ivabradine is slowing of the SA-node and thereby slowing the heart rate. (247) Again, these new options need to be tested specifically in models with different mutations, and in other cardiac diseases which involves increased risk of triggered arrhythmias.

9 Conclusions

The main conclusions of the thesis are:

1. In CPVT1, we confirm the heart rate-independent and essential pro-arrhythmic effects of beta-adrenoceptor stimulation, but the effect of high heart rate was identified as an independent and synergistic contribution.
2. ET reduced ventricular tachycardia episodes in mice with a CPVT1-causative *RyR2* mutation through lower CaMKII-dependent phosphorylation of RyR2 resulting in lower diastolic SR Ca^{2+} release.
3. ET increased aerobic capacity in post-myocardial infarction HF rats and stabilized RyR2-dependent SR Ca^{2+} release, without major alterations in SR Ca^{2+} regulatory proteins.

10 Reference list

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11 Appendix: Paper 1-3

I

PLOS ONE

RESEARCH ARTICLE

Arrhythmia initiation in catecholaminergic polymorphic ventricular tachycardia type 1 depends on both heart rate and sympathetic stimulation

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Abstract

Aims

Catecholaminergic polymorphic ventricular tachycardia type 1 (CPVT1) predisposes to ventricular tachyarrhythmias (VTs) during high heart rates due to physical or psychological stress. The essential role of catecholaminergic effects on ventricular cardiomyocytes in this situation is well documented, but the importance of heart rate *per se* for arrhythmia initiation in CPVT1 is largely unexplored.

Methods and results

Sixteen CPVT1 patients performed a bicycle stress-test. Occurrence of VT triggers, i.e. premature ventricular complexes (PVC), depended on high heart rate, with individual thresholds. Atrial pacing above the individual PVC threshold in three patients did not induce PVCs.

The underlying mechanism for the clinical observation was explored using cardiomyocytes from mice with the *RyR2*-R2474S (RyR2-RS) mutation, which exhibit exercise-induced VTs. While rapid pacing increased the number of Ca^{2+} waves in both RyR2-RS and wild-type ($p<0.05$), β-adrenoceptor (βAR) stimulation induced more Ca^{2+} waves in RyR2-RS ($p<0.05$). Notably, Ca^{2+} waves occurred despite decreased sarcoplasmic reticulum (SR) Ca^{2+} content in RyR2-RS ($p<0.05$), suggesting increased cytosolic RyR2 Ca^{2+} sensitivity.

A computational model of mouse ventricular cardiomyocyte electrophysiology reproduced the cellular CPVT1 phenotype when RyR2 Ca^{2+} sensitivity was increased. Importantly, diastolic fluctuations in phosphorylation of RyR2 and SR Ca^{2+} content determined

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Ca^{2+} wave initiation. These factors were modulated towards increased propensity for arrhythmia initiation by increased pacing rates, but even more by βAR stimulation.

Conclusion

In CPVT1, VT propensity depends on individual heart rate thresholds for PVCs. Through converging data from clinical exercise stress-testing, cellular studies and computational modelling, we confirm the heart rate-independent pro-arrhythmic effects of βAR stimulation in CPVT1, but also identify an independent and synergistic contribution from effects of high heart rate.

1. Introduction

Patients with catecholaminergic polymorphic ventricular tachycardia (CPVT) have an increased risk of sudden cardiac death due to ventricular arrhythmias. The mortality rate in untreated patients is 30–33% by the age of 35.[1] Current treatment options comprise β-adrenoceptor (βAR) antagonists and flecainide.[2–4] In earlier studies, as many as 46% of patients treated with βAR antagonists experienced breakthrough ventricular tachycardias (VT).[5] Flecainide offers effective added protection, but some patients still experience breakthrough VTs even on combined treatment.[2, 6, 7] In such patients, or patients who do not tolerate treatment with βAR antagonists, left cardiac sympathetic denervation can be an option.[8–10] If serious arrhythmic events occur despite optimal medical treatment, an implantable cardioverter defibrillator (ICD) is recommended,[8] but involves a risk of inappropriate shocks that can lead to patient distress and initiate VT and death.[3] Thus, new therapeutic strategies are needed, based on improved mechanistic insight.

Stress testing of patients with CPVT, a central diagnostic strategy, shows a relationship between increasing heart rate and the occurrence of ventricular ectopy.[1, 5] More severe arrhythmias are often observed during high heart rates, such as sustained VTs appearing above a certain heart rate threshold.[1] βAR stimulation has been identified as an important factor for the development of arrhythmias in CPVT, and catecholamines, i.e. adrenaline [11] or isoprenaline (ISO), infusion [12] has been used as a stress test in CPVT. However, the diagnostic polymorphic non-sustained VT was only induced in 31% of patients with mutations pathogenic for CPVT.[5] *In vivo*, βAR stimulation increases heart rate,[13] but also has important non-chronotropic, i.e. heart-rate independent, effects on ventricular cardiomyocytes. On the other hand, increased heart rate has important effects on ventricular cardiomyocytes that are independent of βAR stimulation. Therefore, clarifying the relative importance of the heart rate and sympathetic activity for development of arrhythmias in CPVT could have important implications for diagnostic procedures and treatment strategies.

The focus of this study is CPVT type 1 (CPVT1), caused by mutations in the gene encoding the major intracellular cardiac Ca^{2+} release channel, i.e. the ryanodine receptor 2 (RyR2).[14] *RyR2* mutations in patients with CPVT1 cause pathological Ca^{2+} leak from the sarcoplasmic reticulum (SR) in ventricular cardiomyocytes.[15, 16] Diastolic SR Ca^{2+} leak may lead to delayed afterdepolarization (DAD) and trigger ventricular arrhythmias.[15] Theoretically, βAR stimulation and high heart rate can increase the amplitude of DADs, and promote triggered activity.[17] Accumulating evidence indicates that Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) could be a common mediator for the effects of both heart rate and βAR stimulation.[18, 19] CaMKII-dependent phosphorylation increases RyR2 channel opening

probability, and thus the propensity for increased SR Ca^{2+} leak and arrhythmogenic Ca^{2+} waves.[20] Indeed, inhibition of CaMKII has proved beneficial in models of CPVT1.[19]

We hypothesized that both heart rate and βAR stimulation contribute independently to the development of ventricular arrhythmias in CPVT1. We tested this hypothesis by combining observations from patients, cellular experiments and mathematical modeling.

2. Methods

2.1 Patients and patient data

Patients with genetically confirmed CPVT1 were included through the Department of Cardiology, Oslo University Hospital Rikshospitalet. The study was approved by the Regional Committee for Medical and Health Research Ethics (REC-South-East; REC ID 201772 / 2011–19297), and conformed to the declaration of Helsinki. Written informed consent was obtained from all enrolled patients.

Sixteen patients performed standardized bicycle stress testing using a protocol previously described.[21, 22] Briefly, 12-lead ECGs were recorded during bicycling with increasing workload (Schiller CS-200 Ergo-Spiro, Diacor), starting at 25 W with stepwise increase until exhaustion. One to four tests per patient were included in the study. The threshold heart rate for ventricular arrhythmias in individual patients was defined as the heart rate at which premature ventricular complexes (PVC) occurred as bigeminy, couplets, or VT during stress testing. If patients did not develop any of these arrhythmic events, the threshold was set as the heart rate were single PVCs occurred.

Three patients with ICDs volunteered for an ICD-based pacing protocol following the bicycle stress test. In accordance with approval from the regional Ethical Committee, the pacing procedure was performed as part of the standard follow-up of these patients, and with a minimum of intervention. We wanted to assess the heart rate for start of ventricular arrhythmias before the pacing, to be able to choose the correct rate. Therefore, the exercise stress test had to be performed first and according to standard follow-up protocol. After cessation of the exercise test patients rested in the supine position until recovery of baseline heart rate, and for at least 10 minutes before the pacing procedure was performed as an add-on to their standard ICD control. Electrical pacing through the atrial electrode was performed for 30 s at 5–10 beats per minute (b.p.m.) above the individual threshold heart rate for ventricular arrhythmias identified during the bicycle stress test. A 12-lead ECG was recorded continuously during the ICD-pacing protocol.

2.2 Animal model of CPVT1

This project was approved by the Norwegian National Committee for Animal Welfare under the Norwegian Animal Welfare Act (FOTS ID: 7169, 5669), and conformed to the National Institute of Health guidelines (NIH publication No. 85–23, revised 1996, US). The generation of knock-in mice with a human CPVT1 causative *RyR2*-R2474S (RyR2-RS) mutation used in this study has been described previously.[16]

2.3 Cellular experiments

Mice were anaesthetized in 2% isoflurane inhalation prior to sacrifice by cervical dislocation. Left ventricular cardiomyocytes were isolated using constant flow perfusion of the coronary arteries with collagenase-containing solution as previously described.[23]

Whole-cell Ca^{2+} imaging in field-stimulated cardiomyocytes was performed with a PTI Microscope Photometer D-104G (PhotoMed, Køge, Denmark). Confocal line-scan images

were recorded using a Zeiss *LSM 7* Live confocal microscope (Zeiss Observer Z1, Micro imaging, GmbH, Germany). All experiments were performed at 37˚C and the experimental superfusate was based on a modified Hepes-Tyrode's solution containing (in mM): Hepes 5, NaCl 140, KCl 5.4, $MgCl_2$ 0.5, Glucose 5.5, NaH_2PO_4 0.4, $CaCl_2$ 1. pH was adjusted to 7.4 with NaOH. Fluo-4AM (5 μM, Molecular Probes, Eugene, Oregon, USA) was used to visualize cytosolic Ca^{2+}, and isoprenaline (200 nM, NAF, Norway) for βAR stimulation.

2.4 Western blots

Hearts used for protein analysis were mounted on a modified Langendorff setup, and perfused through the aorta with a 37˚C modified Hepes-Tyrode's solution. The hearts were then paced at 4 or 8 Hz for three min, i.e. the same duration as the protocol for cellular experiments. This frequency was based on pilot experiments, and chosen to allow stable pacing. The frequency of activation was confirmed by simultaneous ECG recordings by electrodes from telemetric ECG transmitters (Data Sciences International, St. Paul, USA). After three min of pacing, the left ventricle was isolated, rapidly frozen in liquid nitrogen, and stored at –80˚C. For βAR stimulation, hearts were perfused with ISO (200 nM) for 1 min.

Western blotting was performed with total protein homogenates from left ventricles, as previously described.[23]

2.5 Computer model

To quantitatively explore the effects of heart rate and βAR stimulation on Ca^{2+} handling in ventricular myocytes, we employed a computational model of mouse ventricular myocyte electrophysiology previously published by Morotti *et al.*[24] This model includes detailed representations of all membrane ion channels, as well as phospholemman, RyR2, the sarcoplasmic reticulum Ca^{2+} ATPase, phospholamban (PLB) and Troponin I. Importantly, this model also includes detailed and dynamic representations of protein kinase A (PKA) and CaMKII activity and their regulation of these ion channels and Ca^{2+} handling proteins. To model RyR2-RS cardiomyocytes, the Morotti computational model was only altered by increasing RyR2 luminal Ca^{2+} sensitivity until the model reproduced Ca^{2+} wave frequency and latency measured in RyR2-RS during cellular experiments.

Briefly, the Morotti RyR2 formulation is an extension of the 4-state model of Shannon *et al.* [25] for which RyR2 SR luminal Ca^{2+} sensitivity is calculated as a sigmoidal function of the luminal Ca^{2+} concentration. This sensitivity can be modulated by the half maximal effective concentration (EC50) for luminal Ca^{2+}, which increases the RyR2 closed-to-open transition rate, and reciprocally reduces the open-to-inactive transition rate. The EC50 was the only parameter we modulated to fit experimental RyR2-RS data. We simulated a range of increased Ca^{2+} sensitivities, from 2–30% above the value in the original Morotti model, to identify the Ca^{2+} sensitivity that best fit the experimental data on the latency of Ca^{2+} wave after pacing and the number of Ca^{2+} waves in the post-pacing period, and remained in qualitative agreement with steady state Ca^{2+} handling. The simulations for RyR2-RS presented in this article were all run with a constant EC50 value, which was decreased by 10% compared to WT.

2.6 Statistics

Results are reported as mean ± standard error of mean (SEM). Ca^{2+} sparks data are reported by density plots using kernel density smoothing with a bandwidth of 0.3 in R software (version 3.0.2, The R Foundation for Statistical Computing). ANOVA or Nested ANOVA analysis with Bonferroni corrections were used as appropriate for analysis of RR-intervals in CPVT1 patients and cellular experiments, except analysis of Ca^{2+} spark frequency for which Poisson

analysis was used to adjust for a skewed distribution. $P<0.05$ was considered statistically significant.

3. Results

3.1 Ventricular arrhythmias in patients with CPVT1 were associated with increased heart rates during bicycle testing, but not during direct pacing

Sixteen patients (39±4 y, 56% women) diagnosed with CPVT1 were included. All patients were positive for CPVT-associated RyR2 mutations, two were probands and 14 were identified as part of family screening. Of these, 11 patients (69%) had symptoms associated with arrhythmias. All patients had been evaluated according to guidelines with echocardiography, and seven patients (44%) also with cardiac MR as part of the initial evaluation.(Table 1).

ECGs were recorded during bicycle stress tests from all patients. When available, we included results from multiple stress tests per patient. No patients exhibited PVCs or ventricular arrhythmias at rest, and the mean RR-interval at baseline was longer than the RR-interval immediately preceding any of the ventricular arrhythmias observed ($p<0.05$, Fig 1A–1C). This illustration of the heart rate dependence of ventricular arrhythmias in CPVT1 was seen both in untreated patients and in patients treated with a beta-adrenoceptor antagonist (Fig 1C).

In addition to the sixteen patients included based on available data from bicycle stress testing, we included three patients harboring an ICD implanted due to CPVT1 (Table 1). To test the importance of heart rate, we performed atrial pacing at rest in these patients. The pacing protocol was performed following bicycle testing after complete recovery to baseline heart rate. Atrial pacing was performed at 5–10 b.p.m. above the threshold for occurrence of PVCs during the bicycle stress test. None of the patients developed PVCs during pacing through the ICD (Fig 1D).

3.2 βAR stimulation revealed an increased propensity for arrhythmogenic Ca^{2+} release events in RyR2-RS mouse left ventricular cardiomyocytes

The propensity for diastolic Ca^{2+} waves in RyR2-RS and WT left ventricular cardiomyocytes was measured in a 10 s period after stable Ca^{2+} transients, which had been induced by field stimulation for 30 s (Fig 2A). In absence of βAR stimulation, progressive increase of the pacing frequency raised the number of Ca^{2+} waves in both RyR2-RS and WT ($p<0.05$), but did not reveal any differences between RyR2-RS and WT (Fig 2B). βAR stimulation, however, resulted in an increased number of Ca^{2+} waves in RyR2-RS compared to WT both at 0.5 and 4 Hz pacing ($p<0.05$, Fig 2B).

Another measurement of Ca^{2+} wave propensity is the time to occurrence of the first Ca^{2+} wave upon secession of pacing, i.e. post-pacing Ca^{2+} wave latency. This period decreased with pacing frequency in both RyR2-RS and WT ($p<0.05$), but was shorter in RyR2-RS than WT at 4 and 8 Hz both in the absence and presence of βAR stimulation ($p<0.05$, Fig 2C).

To test the dose-response relationship between βAR stimulation and the number of Ca^{2+} waves in the post-pacing period, cardiomyocytes were exposed to 0, 2, 20 and 200 nM ISO (4 Hz pacing, Fig 2D). Increasing ISO concentrations resulted in increased frequency of Ca^{2+} waves in both WT and RyR2-RS ($p<0.05$). Overall, the frequency of Ca^{2+} waves was higher in RyR2-RS than WT across ISO concentrations ($p<0.05$).

To further characterize diastolic SR Ca^{2+} release in RyR2-RS and WT, Ca^{2+} sparks were recorded by confocal microscopy (Fig 3A and 3B). In the absence of βAR stimulation, the propensity for Ca^{2+} sparks was low in both RyR2-RS and WT (Fig 3C), while during βAR stimulation the number of Ca^{2+} sparks increased in both groups and was higher in RyR2-RS compared to WT ($p<0.05$, Fig 3D).

Table 1. Patient overview.

Patient	Test number	Gender	Age at diagnosis	Family member vs. Proband	Mutation	Symptoms	Family history	Comorbidities	Imaging	Medication	ICD	Age at-stress test	No βAR antagonist		With βAR antagonist	
													Baseline	RR interval before first arrhythmic event	Baseline	RR interval before first arrhythmic event
1	I	Woman	43	Family member	G2337V 46 RyR2	Palpitations	SCD daughter 13y; SCD brother 21y	No	Normal echo	No	No	43	880	440		
	II					no				Metoprolol succinate 100 mg x 1	No	44			980	
2	I	Woman	38	Family member	G4671V 97 RyR2	Syncope while swimming	Son with cardiac symptoms	No	Normal echo; Normal CMR	No	No	38	940	340		
	II					No				Metoprolol succinate 100 mg x 1	No	39			820	390
3	I	Woman	29	Family member	G2337V 46 RyR2	Palpitations	SCD in family member with CPVT	No	Normal echo	No	No	28		360		
	II					No				Metoprolol succinate 100 mg x 1	No	29			1040	440
4	I	Woman	50	Family member	G2337V 46 RyR2	No	SCD son 24y	No	Normal echo; normal CMR	No	No	49				
	II					No				Metoprolol succinate 100 mg x 1	No	49				
	III					No				Metoprolol succinate 150 mg x 1	No	50				
5	I	Woman	58	Family member	G4671V 97 RyR2	Palpitations	Daughter and grandchild with CPVT	No	Mild mitral regurgitation on echo	No	No	56	980	440		
6	I	Man	56	Family member	G2337V 46 RyR2	No	SCD brother 10y; SCD brother 44y	No	Medium mitral regurgitation and mild aortic regurgitation on echo	Metoprolol succinate 100 mg x 1	No	55			1040	540
	II					No				Metoprolol succinate 150 mg x 1	No	56			1300	
	III					No				Metoprolol succinate 150 mg x 1	No	57			1320	560

(*Continued*)

Table 1. (Continued)

Patient	Test number	Gender	Age at diagnosis	Family member vs. Proband	Mutation	Symptoms	Family history	Comorbidities	Imaging	Medication	ICD	Age at-stress test	No βAR antagonist		With βAR antagonist	
													Baseline	RR interval before first arrhythmic event	Baseline	RR interval before first arrhythmic event
7	I	Man	39	Family member	G2337V 46 RyR2	No	SCD several family members	No	Normal echo.; normal CMR	No	No	39	1040	420		
	II					No				Metoprolol succinate 50 mg x 1	No	40			940	430
	III					No				Carvedilol 25 mg x1	No	41			960	360
	IV					No				Carvedilol 25 mg x1	No	41			1180	400
8	I	Man	41	Family member	G2337V 46 RyR2	No	SCD several family members	No	Normal echo	No	No	41	840	460		
9	I	Man	36	Family member	G2337V 46 RyR2	Syncope during physical activity	SCD several family members	No	Normal echo	No	No	36	1200	440		
	II					No				Metoprolol succinate 100 mg x 1	No	36			1200	500
	III					No				Metoprolol succinate 100 mg x 1	No	37			1060	540
10	I	Woman	66	Family member	G2337V 46 RyR2	No	SCD grandchild with CPVT	Hypertention	Small aortic regurgitation on echo	Metoprolol succinate 50 mg x 2	No	68			1260	480
11	I	Man	22	Family member	G2337V 46 RyR2	No	SCD several family members	No	Normal echo	Metoprolol succinate 50 mg x 1	No	23	1100			
12	I	Man	19	Family member	G2337V 46 RyR2	No	SCD father and several family members	Allergies	Normal echo	No	No	19				
13	I	Man	35	Family member	G2337V 46 RyR2	Palpitations	SCD several family members	Hypothyroidism	Normal echo	Levothyroxine 50 mcg x 1	No	35	1260	440		
	II					No				Metoprolol succinate 100 mg x 1, Levothyroxin	No	35			1080	500

(*Continued*)

Table 1. (Continued)

Patient	Test number	Gender	Age at diagnosis	Family member vs. Proband	Mutation	Symptoms	Family history	Comorbidities	Imaging	Medication	ICD	Age at-stress test	No βAR antagonist		With βAR antagonist	
													Baseline	RR interval before first arrhythmic event	Baseline	RR interval before first arrhythmic event
14	I	Woman	54	Family member	G2337V 46 RyR2	Palpitations	SCD in family member with CPVT	Hypothyroidism	Normal echo	Levothyroxine	No	54	1400			
	II					No				Metoprolol succinate 50 mg x 1, Levothyroxine	No	54			1960	640
	III					Palpitations				Metoprolol succinate 50 mg x 1, Levothyroxine	No	56			1760	
15	I	Man	13	Proband	G4671V 97 RyR2	Ventricular fibrillation during svimming		No	Small aortic regurgitation on echo.; Normal CMR	Metoprolol succinate 100 mg x 2	No	17	680			
16	I	Woman	18	Proband	R176Q 8 RyR2	Multiple syncopes	SCD brother	No	Normal echo	Nadolol 40 mg x 1	No	26			1200	560
Average													1032 ± 68	418 ± 15*	1194 ± 74	488 ± 22*
Patients with ICD included in pacing protocol																
17		Woman	39	Family member	G2337V 46 RyR2	Four episodes of syncope during swimming and physical activity	SCD son 10y	No	Normal echo.; normal CMR	Metoprolol succinate 50 mg x 1	Yes	48				
18		Man	13	Proband	F4176S 90 RyR2	Multiple syncopes		No	Normal echo	Nadolol 80 mg + 60 mg, Flecainide 100 mg x 2, Methylphenidate	Yes	18				
19		Man	15	Family member	R176Q 8 RyR2	Multiple syncopes	Sister with CPVT	No	Normal echo, normal CMR	Metoprolol succinate 75 mg x 2, Flecainide 100 mg x 2	Yes	20				

SCD, sudden cardiac death; CMR, cardiac magnetic resonance imaging.

*$p<0.00001$ vs. baseline.

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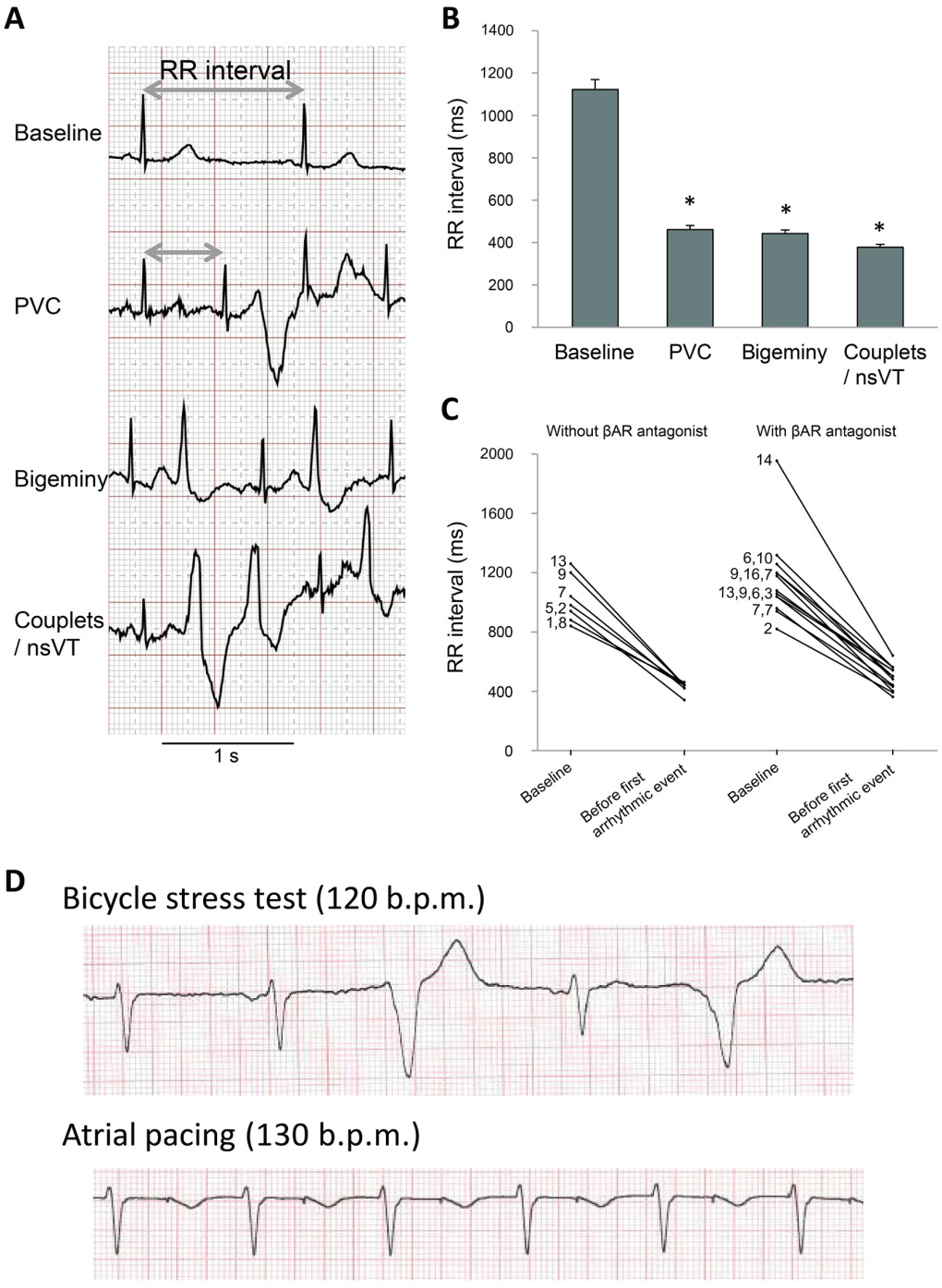

Fig 1. Bicycle stress testing showed the heart rate-dependence of arrhythmias in patients with CPVT1, while atrial pacing did not induce arrhythmias. (A) ECG tracings from a patient with CPVT1 recorded during a bicycle stress test. RR-interval was measured before PVCs occurred. (B) Bar graphs of RR-intervals of sinus beats in stable sinus rhythm (baseline), and immediately before the occurrence of isolated PVCs, PVCs in bigeminy, and couplets or nsVT, respectively. Data from 31 bicycle stress tests performed by 16 patients with CPVT1. *$p<0.05$ compared to baseline with one-way ANOVA with Bonferroni correction. (C) Individual plots of RR-intervals of patients who developed arrhythmias during the stress test. RR-intervals at baseline and before the first arrhythmic event are shown, both in absence and in presence of βAR antagonists. (D) ECG tracings from a patient with CPVT1 recorded during a bicycle stress test (upper panel) with PVCs in bigeminy at 120 beat per minute (b.p.m.), and at rest during pacing through an atrial lead with no PVCs at 130 b.p.m. (lower panel).

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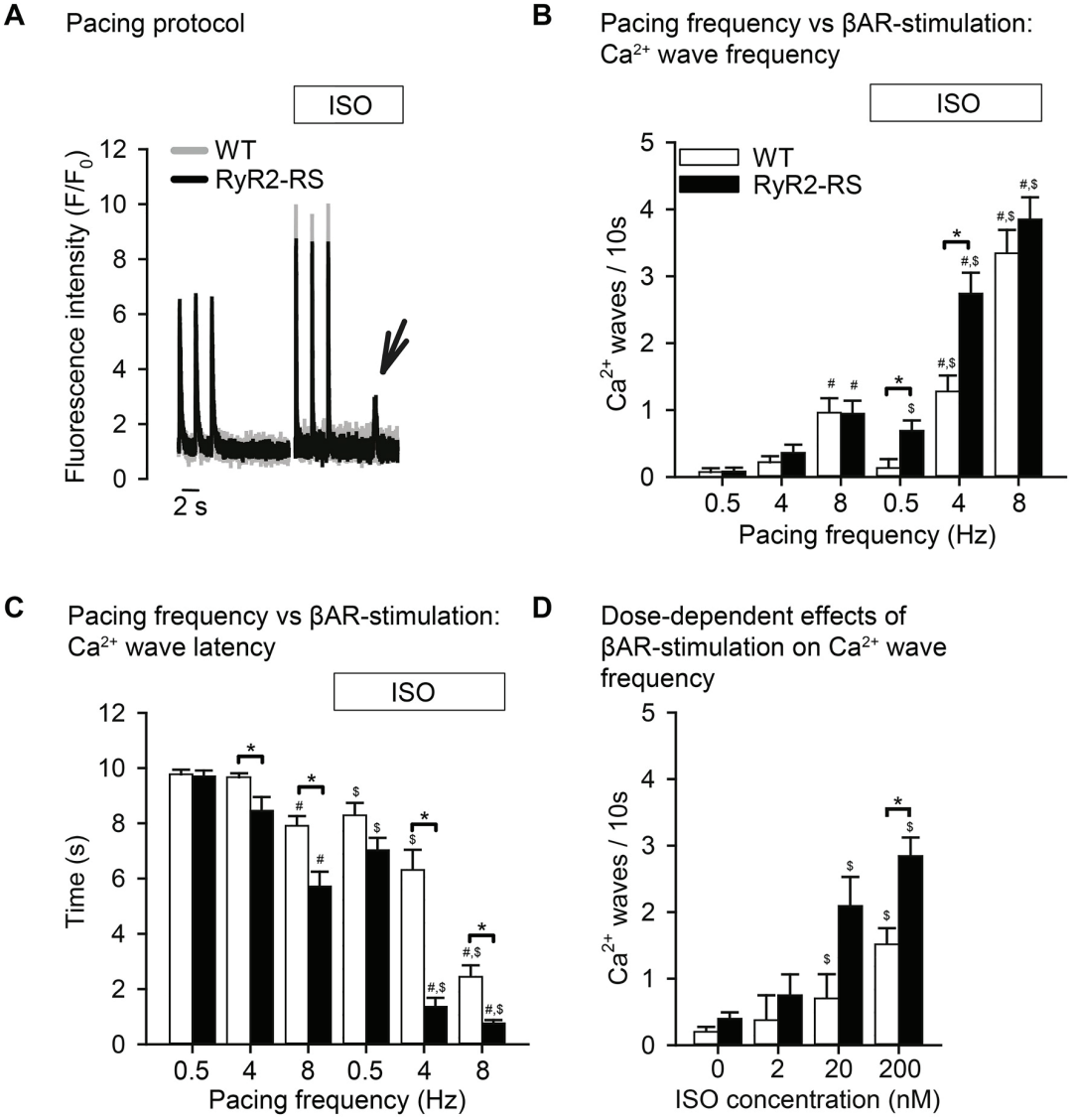

Fig 2. High pacing frequency induced Ca^{2+} waves in RyR2-RS mouse left ventricular cardiomyocytes, but βAR stimulation was necessary to reveal increased propensity compared to WT. (A) Tracings of whole-cell Ca^{2+} fluorescence showing Ca^{2+} transients and Ca^{2+} waves (arrow) during 0.5 Hz pacing by field stimulation in absence and presence of stimulation of βARs with ISO. (B) Bar graphs showing the mean frequency of Ca^{2+} waves in RyR2-RS and WT cardiomyocytes in a 10 s post-pacing period after 0.5, 4 and 8 Hz pacing in absence and presence of ISO, respectively. Analyzed by Nested ANOVA with data from 9 RyR2-RS and 8–15 WT mice per bar (29–55 cells per result). (C) Bar graphs showing the mean Ca^{2+} wave latency in RyR2-RS and WT cardiomyocytes after different pacing frequencies, in absence and presence of ISO. Analyzed by Nested ANOVA with data from 9–16 RyR2-RS and 10–21 WT mice per bar (28–70 cells per result). (D) Bar graph showing the mean frequency of Ca^{2+} waves in a 10 s period after 4 Hz pacing in 0, 2, 20 and 200 nM ISO. Analyzed by Nested ANOVA with data from 3–13 RyR2-RS and 3–18 WT mice (8–65 cells per result). $^{*}p<0.05$ RyR2-RS vs WT, $^{\#}p<0.05$ vs 0.5 Hz in the same conditions (+/- ISO), $^{\$}p<0.05$ +ISO vs–ISO for the same genotype and frequency.

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3.3 SR Ca^{2+} content and threshold for Ca^{2+} waves were lower in RyR2-RS than WT

The effects of pacing and βAR stimulation on Ca^{2+} release and removal was further investigated by characterization of key aspects of Ca^{2+} handling in isolated left ventricular

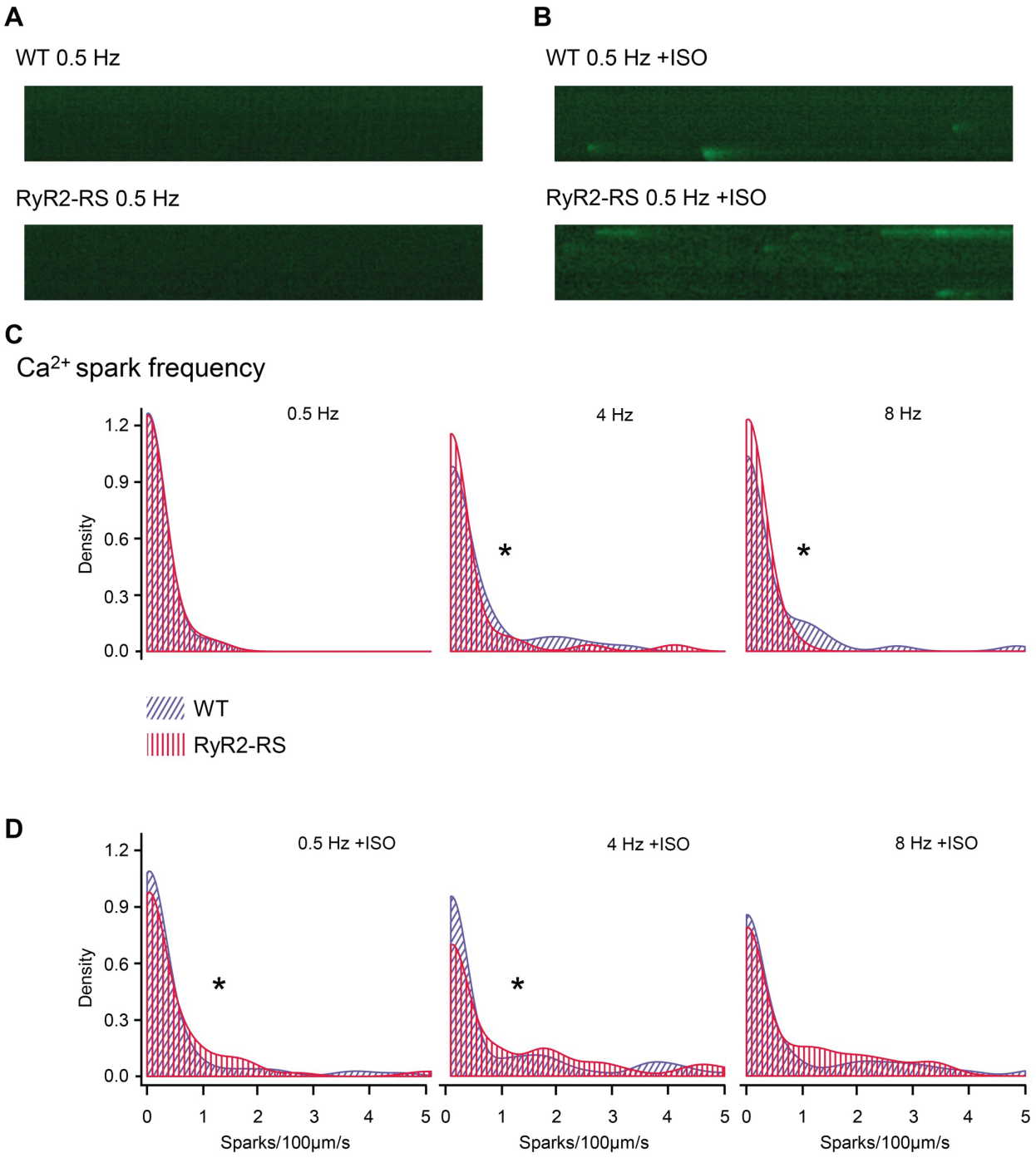

Fig 3. High pacing frequency induced Ca^{2+} sparks in RyR2-RS mouse left ventricular cardiomyocytes, but βARstimulation was necessary to reveal increased propensity compared to WT. (A) Line scan confocal imaging in absence of stimulation of βARs with ISO after 0.5 Hz pacing in WT (upper panel) and RyR2-RS (lower panel). (B) Line scan confocal imaging in presence of ISO after 0.5 Hz pacing in WT (upper panel) and RyR2-RS (lower panel). (C) and (D) Density plots illustrating the distribution of number of cells with 0–5 Ca^{2+} sparks per 100 μm per second, occurring after 0.5, 4 and 8 Hz pacing in absence and presence of ISO, respectively. Higher density means more cells. The legend shows how different patterns represent WT or RyR2-RS, respectively. Results from Poisson analysis of data from 13 RyR2-RS mice (47 cells) and 15 WT mice (62 cells), $^{*}p<0.05$.

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cardiomyocytes. This was studied by pacing and caffeine induced Ca^{2+} transients in RyR2-RS and WT (Fig 4A–4D). In absence of βAR stimulation, RyR2-RS developed higher Ca^{2+} transient amplitudes compared to WT at 4 Hz, while at 0.5 and 8 Hz, no significant differences were found (Fig 4E). Following βAR stimulation, however, the Ca^{2+} transient amplitude was lower in RyR2-RS compared to WT at all pacing frequencies ($p<0.05$, Fig 4E). In line with this, SR Ca^{2+} content in the absence of βAR stimulation was not significantly different in RyR2-RS and WT across pacing frequencies (Fig 4F), while following βAR stimulation, SR Ca^{2+} content was lower in RyR2-RS than WT overall ($p<0.05$), and at both 0.5 and 4 Hz (Fig 4F, $p<0.05$). These differences could not be explained by refilling of the SR, as cytosolic Ca^{2+} removal rate, a key determinant for the Ca^{2+} transient amplitude and SR Ca^{2+} content, was not significantly different between RyR2-RS and WT (Fig 4G).

Next, threshold SR Ca^{2+} content, defined as SR Ca^{2+} content at which Ca^{2+} waves occurred, was assessed (Fig 4D and 4H). Overall, Ca^{2+} waves developed at a lower SR Ca^{2+} content in RyR2-RS than in WT both in the absence ($p<0.05$) and presence of βAR stimulation ($p<0.05$).

3.4 Measurements of abundance of Ca^{2+} handling proteins did not reveal differences between RyR2-RS and WT

The abundance of key Ca^{2+} handling proteins and phosphoproteins were quantified in left ventricular tissue from Langendorff perfused and paced hearts (Fig 5 and S1 Fig). The only observed difference between the RyR-RS and WT was higher abundance of CaMKII phosphorylated at threonine286, i.e. autophosphorylated CaMKII, in RyR2-RS compared to WT at 8 Hz in the absence of βAR stimulation ($p<0.05$, Fig 5A). No significant differences between RyR2-RS and WT were observed with regard to the abundance of RyR2 phospho-serine2808, RyR2 phospho-serine2814, PLB phospho-serine16 or PLB phospho-threonine17 at 4 or 8 Hz pacing (Fig 5B–5E). SERCA2a abundance was also similar in RyR2-RS and WT (Fig 5F). Importantly for quality control, βAR stimulation increased PLB phospho-serine16 in both RyR2-RS and WT ($p<0.05$, Fig 5B and 5D).

3.5 Computer simulations support that pacing rate and βAR stimulation have independent effects on the propensity for Ca^{2+} waves in both RyR2-RS and WT

A computational model of mouse ventricular cardiomyocyte electrophysiology and ion homeostasis was employed to deconvolve the factors underlying the effects of pacing frequency and βAR stimulation on Ca^{2+} wave propensity.[24] With a 10% increase in RyR2 luminal Ca^{2+} sensitivity, the model reproduced the effects of pacing and βAR stimulation on Ca^{2+} wave frequency and latency in a 10 s post pacing period (S2 Fig). The model allowed a continuous readout of intracellular Ca^{2+}, SR Ca^{2+} content, CaMKII activity, and level of CaMKII-dependent phosphorylation of RyR2 at serine 2814. The following observations were made regarding the development of Ca^{2+} waves in the post-pacing period (Fig 6): First, at 0.5 Hz βAR stimulation was necessary for Ca^{2+} waves to occur in both RyR2-RS and WT (Fig 6A, left vs. right upper panels). Second, when all effects of CaMKII was removed or RyR2 phosphorylation level was kept at baseline, i.e. without effects of pacing or βAR stimulation, Ca^{2+} wave propensity decreased in both RyR2-RS and WT (Fig 6A, red and green lines). Third, higher pacing frequency increased the propensity for Ca^{2+} waves by increased activation of CaMKII and phosphorylation of RyR2 (Fig 6B, left vs right panels). However, higher frequency also increased the propensity for Ca^{2+} waves by increasing SR Ca^{2+} content, but only in WT (Fig 6B). Fourth, the main effect of CaMKII on Ca^{2+} wave propensity depended on the time after cessation of pacing: Early after pacing, CaMKII activity and RyR2 phosphorylation were high,

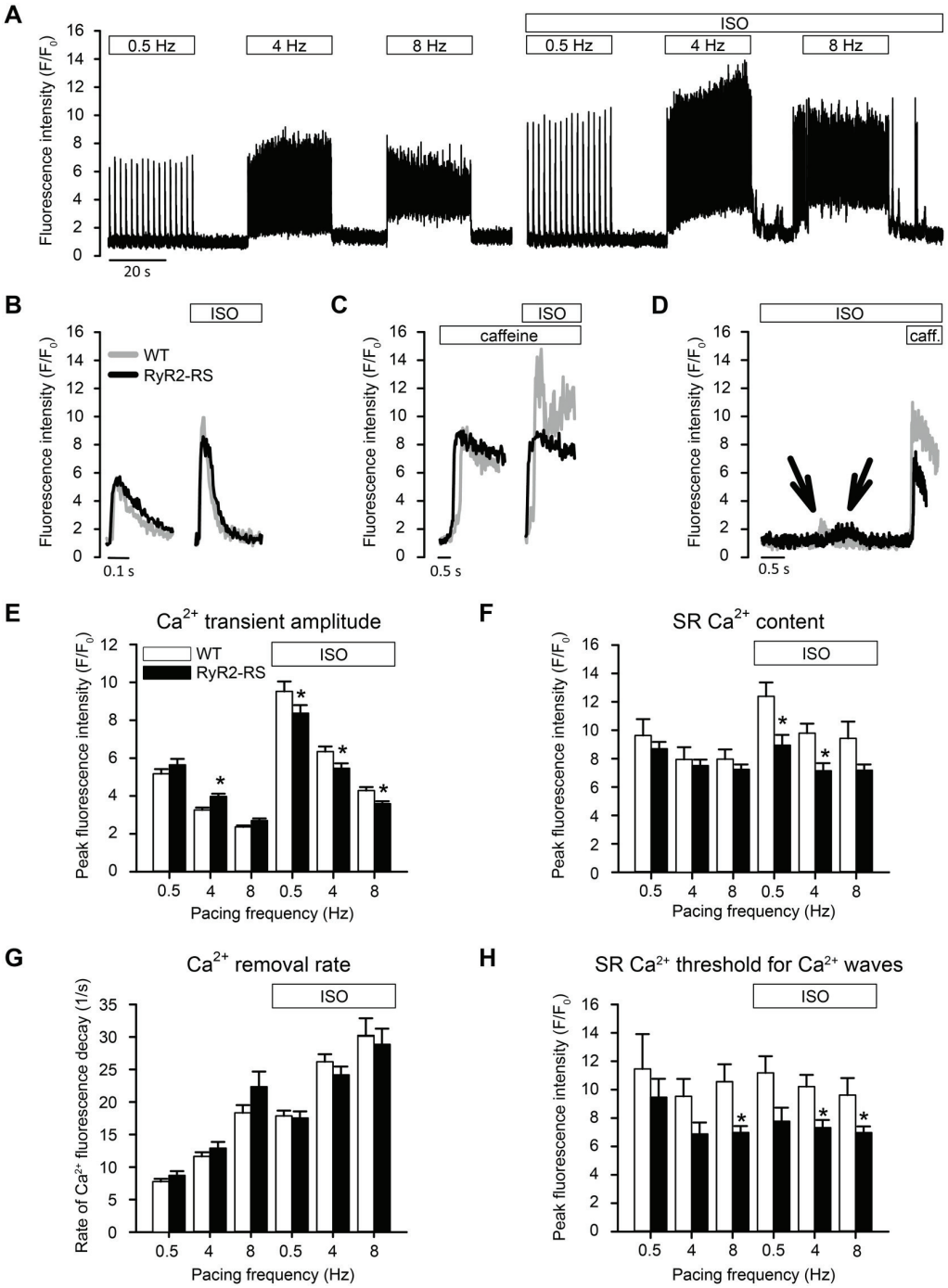

Fig 4. Ca^{2+} handling characteristics in RyR2-RS left ventricular cardiomyocytes indicated increased RyR open probability and lower threshold for diastolic Ca^{2+} release. (A) A whole-cell Ca^{2+} fluorescence tracing showing the experimental protocol with 0.5, 4 and 8 Hz pacing at baseline and during βAR stimulation. (B) Tracings of whole-cell Ca^{2+} fluorescence showing Ca^{2+} transients during 0.5 Hz pacing in absence and presence of ISO. (C) Tracings of whole-cell Ca^{2+} fluorescence showing caffeine-elicited Ca^{2+} release after 0.5 Hz pacing. Peak fluorescence intensity was used for measurements of SR Ca^{2+} content. Caffeine was added immediately after the last stimulated Ca^{2+} transient. (D) Tracings of whole-cell Ca^{2+} fluorescence showing caffeine-elicited Ca^{2+} release at the time of occurrence of Ca^{2+} waves after 4 Hz stimulation. Caffeine was added immediately after the occurrence of a Ca^{2+} wave. This protocol was used for measurements of threshold SR Ca^{2+} content. Peak fluorescence intensity was used for measurements of SR Ca^{2+} content. (E) Bar graphs

showing mean Ca^{2+} transient amplitude at different pacing frequencies in absence and presence of ISO. Analyzed by Nested ANOVA with data from 8–10 RyR2-RS mice and 7–19 WT mice per bar (29–58 cells per result). (F) Bar graphs showing mean SR Ca^{2+} content at different pacing frequencies in absence and presence of ISO. Analyzed by Nested ANOVA with data from 7–10 RyR2-RS mice and 4–11 WT mice per bar (20–26 cells per result). (G) Bar graphs showing mean decay rates of the Ca^{2+} transients at different pacing frequencies in absence and presence of ISO. Analyzed by Nested ANOVA with data from 8–9 RyR2-RS mice and 7–19 WT mice per bar (29–58 cells per result). (H) Bar graphs showing mean threshold SR Ca^{2+} content at different pacing frequencies in absence and presence of ISO. Analyzed by Nested ANOVA with data from 6–7 RyR2-RS mice and 3–5 WT mice per bar (8–20 cells per result), except 0.5 Hz, at which a meaningful threshold was not obtained since very few cells exhibit Ca^{2+} waves. For this frequency, bar graphs represent data from 2 mice (3 cells) in each group. $^{*}p<0.05$ RyR2-RS vs WT.

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allowing Ca^{2+} waves to initiate at a low SR Ca^{2+} content. Ca^{2+} waves occurred earlier in RyR2-RS because the SR Ca^{2+} threshold for Ca^{2+} waves was intrinsically lower than in WT, i.e. the SR Ca^{2+} content at the time of the first Ca^{2+} wave was lower in RyR2-RS (6A-C). Later in the diastolic period, the degree of RyR2 phosphorylation decayed more rapidly than the SR Ca^{2+} refilling, and further Ca^{2+} waves could only be generated by increased SR Ca^{2+} content caused by reduced CaMKII-dependent RyR2 phosphorylation (Fig 6C), and subsequent reduced SR Ca^{2+} leak. With removal of CaMKII-dependent effects on Ca^{2+} handling or by keeping RyR2 phosphorylation level at the baseline for the model, the threshold SR Ca^{2+} content for waves was high even in the early phase of diastole, and time for refilling dominated the Ca^{2+} wave latency (Fig 6A and 6C).

4. Discussion

Our study confirms that ventricular arrhythmias in patients with CPVT1 are associated with increasing heart rate during exercise testing, as previously reported.[1, 21] However, comparisons of bicycle testing to ICD pacing indicate that increased heart rate by itself is not sufficient to induce arrhythmias. Experiments with ventricular cardiomyocytes corroborated this conclusion: while high pacing frequencies increased the number of Ca^{2+} waves in both RyR2-RS and WT, βAR stimulation was necessary to reveal the increased propensity for Ca^{2+} waves associated with CPVT1 in RyR2-RS. Computer simulations of CPVT1 cardiomyocytes further strengthened these findings, and show that although higher pacing frequency promotes Ca^{2+} wave development, the effects of βAR stimulation on SR Ca^{2+} release dynamics are necessary to allow increased propensity for Ca^{2+} waves during high pacing frequencies in RyR-RS cardiomyocytes.

Previous studies have established that βAR stimulation increases the degree of SR Ca^{2+} leak and the propensity for arrhythmias in CPVT1,[26] while the effect of pacing frequency *per se* has not yet been studied in this condition. Based on previous studies, we had reason to believe that frequency could promote SR Ca^{2+} leak, and that this could be partly CaMKII-dependent: In ventricular cardiomyocytes, both high heart rate and βAR stimulation increases the activity of CaMKII, which has been shown to increase SR Ca^{2+} leak.[18, 19] The mechanism for activation of CaMKII during increased pacing frequencies is high cytosolic Ca^{2+} concentration.[27] The importance of CaMKII-dependent SR Ca^{2+} leak in CPVT1 is further indicated by the fact that CaMKII-inhibition by KN-93 or autocamtide-1 related inhibitory peptide reduced spontaneous Ca^{2+} release in ventricular cardiomyocytes from mice with the CPVT1-causative *RyR2*-R4496C mutation.[19] Our results support an important role for CaMKII in RyR2-RS, although the mechanism is somewhat more complex than previously hypothesized.

SR Ca^{2+} leak is highly dependent on SR Ca^{2+} content. In our study, SR Ca^{2+} content did not change with increasing pacing frequency or βAR stimulation in RyR2-RS cardiomyocytes. However, compared to WT, Ca^{2+} transient amplitude in RyR2-RS went from equal or higher without βAR stimulation, to lower during βAR stimulation. This is in contrast to findings in

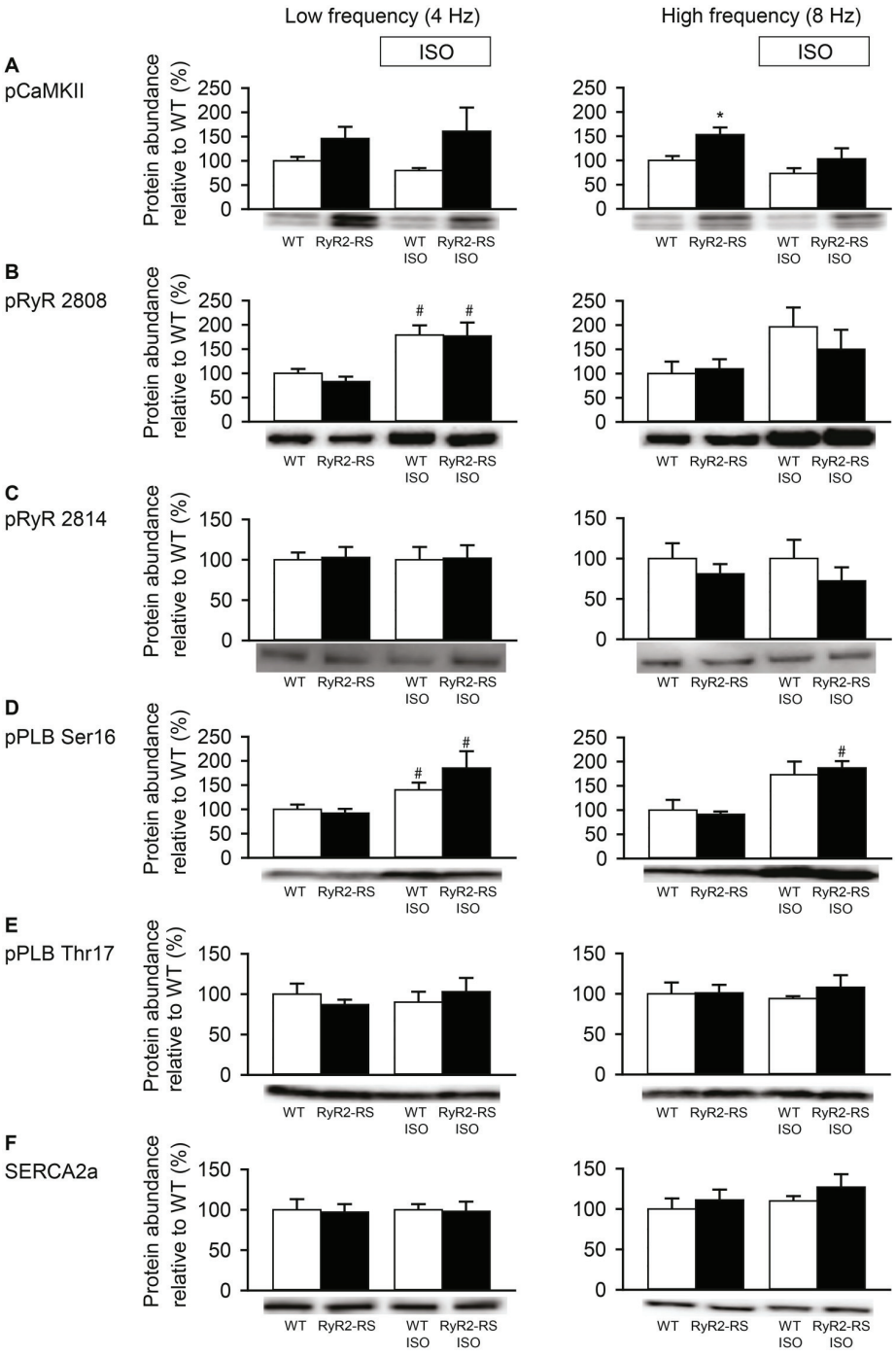

Fig 5. Analysis of key phosphoproteins did not show any differences between RyR2-RS and WT. Protein abundance was analyzed after 4 and 8 Hz pacing in absence and presence of ISO. (A) CaMKII phospho-threonine286 (pCaMKII), (B) RyR2 phospho-serine-2808 (pRyR2808), (C) RyR2 phospho-serine-2814 (pRyR 2814), (D) PLB phospho-serine 16 (pPLB-Ser16), (E) PLB phospho-threonine 17 (pPLB-Thr17), (F) SERCA2a. The graphs show mean protein abundance in 6 hearts from each group. Western blot results were normalized to WT (in the absence of ISO stimulation) at 4 and 8 Hz. $^{*}p<0.05$, #$p<0.05$ vs. baseline in absence of ISO with Student's T-test for unpaired data.

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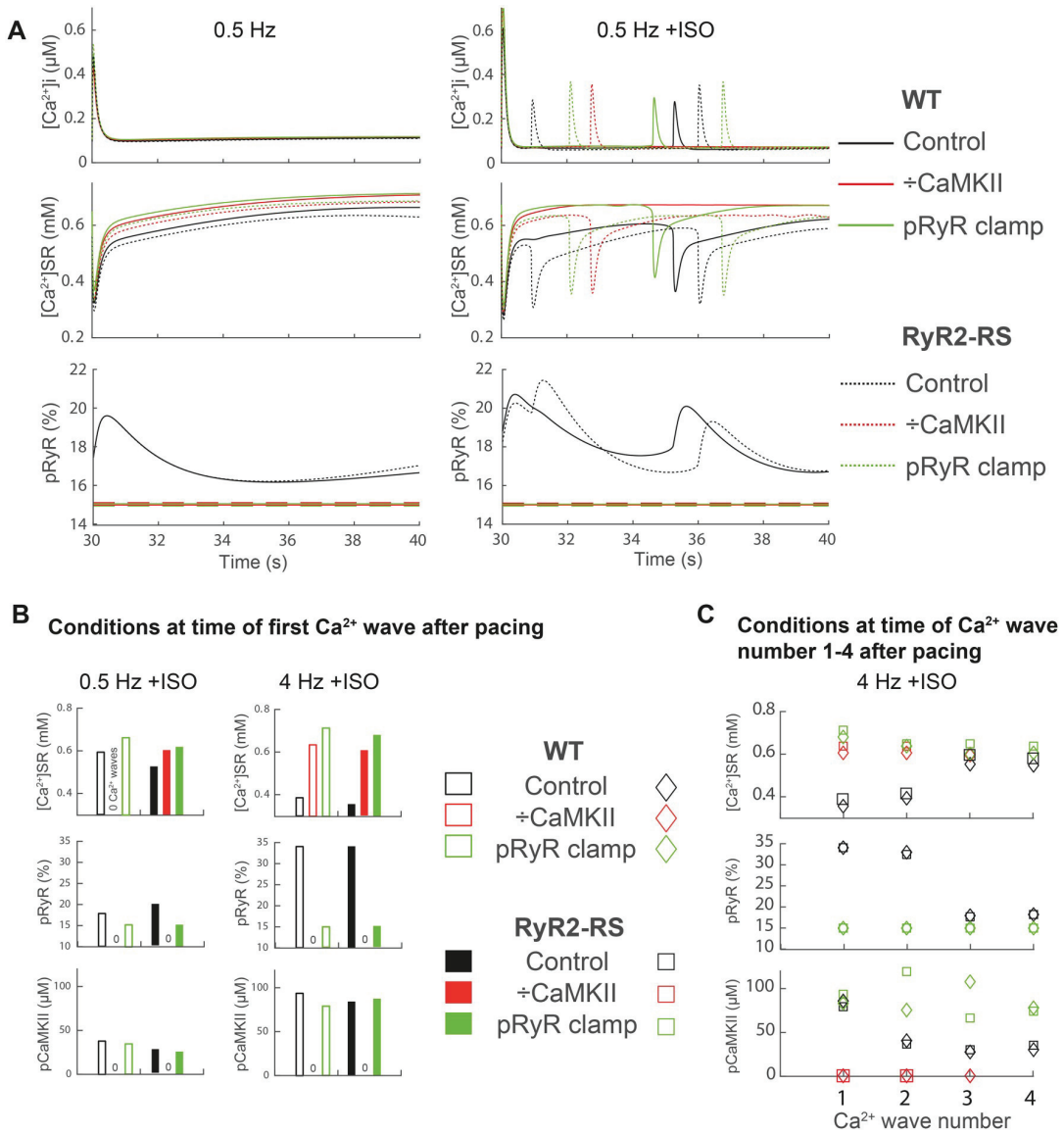

Fig 6. Computational modelling elucidated the interaction between pacing frequency and βAR stimulation in Ca^{2+} wave development. (A) Modelled representation of whole-cell intracellular Ca^{2+} ($[Ca^{2+}]_i$) and SR Ca^{2+} content ($[Ca^{2+}]_{SR}$), as well as fluctuations in the level of RyR2 phosphorylation (pRyR) relative to a quiescent myocyte, in a 10 s period after pacing at 0.5 Hz in absence (left panel) and presence of ISO (right panel). Similar simulations were performed with 4 Hz pacing (not shown). The impact of CaMKII and phosphorylation of RyR2 was tested by omitting CaMKII from the model entirely (÷CaMKII), and by holding RyR2 phosphorylation to a set level found in quiescent cells in absence of ISO (RyR P-clamp). (B) SR Ca^{2+} content ($[Ca^{2+}]_{SR}$), level of RyR2 phosphorylation (pRyR), and abundance of autophosphorylated CaMKII (pCaMKII) at the time of occurrence of the first Ca^{2+} wave after 0.5 and 4 Hz pacing in absence and presence of ISO. Note that after 0.5 Hz in absence of ISO no Ca^{2+} waves occurred in WT. (C) SR Ca^{2+} content, level of RyR2 phosphorylation (RyR-P), and CaMKII activity at the time of the first and subsequent Ca^{2+} waves in a 10 s period after 4 Hz pacing.

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RyR2-R4496C cardiomyocytes in which Ca^{2+} transient amplitude was not changed by βAR stimulation.[19] One explanation for our findings is that the RyR2 is slightly sensitized even in absence of βAR stimulation, resulting in an increased fractional release even at baseline. Indeed, SR Ca^{2+} threshold for Ca^{2+} waves in RyR2-RS was lower even in absence of βAR

stimulation. Thus, one interpretation of our results is that when βAR stimulation is added, SR Ca^{2+} leak increases more than Ca^{2+} homeostatic mechanisms can compensate for, resulting in decreased SR Ca^{2+} content and Ca^{2+} transient amplitude.

We used the computational model to further elucidate the effects of βAR stimulation: The model shows that in absence of βAR stimulation, the rate of SR Ca^{2+} refilling is insufficient to increase the propensity for Ca^{2+} waves. However, in presence of βAR stimulation, SR Ca^{2+} refilling in combination with a decreased threshold for SR Ca^{2+} release is sufficiently fast for early initiation of Ca^{2+} waves. These results are in line with conclusions from the *RyR2*-R4496C CPVT1 mouse model,[26].

Still, two possible mechanisms for the increased number of Ca^{2+} waves seen during βAR stimulation: either that βAR stimulation is necessary to increase SR Ca^{2+} content sufficiently for release, or that such stimulation further destabilizes RyR2 and thereby decreases the SR Ca^{2+} threshold for Ca^{2+} release. Our experimental results did not show a change in this threshold in RyR2-RS cardiomyocytes during βAR stimulation compared to baseline. This could indicate that the main effect of βAR stimulation is to increase SR Ca^{2+} content sufficiently for release. Interestingly, this interpretation is partly supported by our computational model: The major observation across simulations was that the propensity for Ca^{2+} waves to occur depended most critically on two factors that vary in time during the diastolic interval. The first factor is the degree of CaMKII phosphorylation at RyR2. This is because, in this model, increasing CaMKII-dependent phosphorylation of RyR2 reduces the threshold SR Ca^{2+} load for a Ca^{2+} wave. The degree of phosphorylation at any time after a beat depends on both the peak level achieved during pacing, and the rate of dephosphorylation in the period after the beat. The second factor is the rate at which SR Ca^{2+} load is restored during the diastolic interval. Because RyR2 dephosphorylation dynamically increases the threshold SR Ca^{2+} content after each beat, while the SR is simultaneously refilling with Ca^{2+}, it is the combination of these two dynamic effects that determines when a Ca^{2+} wave will occur in this model. βAR stimulation promotes Ca^{2+} waves because it both increases RyR phosphorylation by CaMKII, and dramatically increases the rate of SR Ca^{2+} refilling. Increased pacing frequency also exaggerates both of these effects, but more modestly.

A potentially important observation from our computational model with regard to diastolic Ca^{2+} release is that the effects of CaMKII may be highly dynamic in cardiac myocytes, even during an individual cardiac contraction-relaxation cycle, which might explain why an increase in CaMKII-dependent phosphorylation of RyR2-Ser2814 during ISO stimulation was not detected by immunoblotting. However, changes in RyR2-Ser2814 phosphorylation have been well documented in chronic disease models by previous studies.[18] While the relevance of the observations made from pause-induced release experiments (and simulations) to clinical exercise stress tests is limited, the finding that RyR2 phosphorylation is important in early diastole may help to explain why blockade of CaMKII alleviates the pro-arrhythmia associated with CPVT1-causative mutations.[19]

The discussion above illustrates the complexity of Ca^{2+} homeostasis in ventricular cardiomyocytes at the core of arrhythmia development in CPVT1. However, the complete understanding of arrhythmia development even in this monogenic disease requires even further levels of complexity as both βAR stimulation and heart rate also affects the propensity for DADs to trigger action potentials and the propensity for development of VT.[16, 28] These aspects are beyond the scope of our study. Also important are electrophysiological differences between the human and mouse heart for interpretation of our data: In humans, SR Ca^{2+} content increases with increasing pacing frequencies, contributing to a positive force-frequency relationship; in contrast, mice exhibit a less steep or no increase in SR Ca^{2+} content during increased pacing frequencies.[29] Furthermore, due to a 10-fold faster heart rate and shorter

action potentials the mouse is different from the human heart. To capture spontaneous Ca^{2+} release events occurring during diastole in isolated myocytes, experiments were performed at artificially slow pacing frequencies compared to in vivo heart rates for mice. Because relatively slow pacing can affect the probability of these events, further validation in human cardiomyocytes and in vivo models is warranted.

In our study, direct observations of heart rate effects in patients were made in the ICD-based pacing procedure, which indicated that increased heart rate *per se* was not sufficient to induce arrhythmias. However, the pacing protocol have important limitations: F.ex., during the pacing protocol, heart rate was increased for a shorter time than during the bicycle exercise test. We can only speculate that this could affect CaMKII activation. Our results show that the untangling of the effects of cathecolamines and heart rate should be further pursued in future studies, including extended and comprehensive pacing procedures.

In conclusion, our clinical and experimental data, as well as computational modelling, show that increased heart rate and heart rate-independent effects of βAR stimulation on ventricular cardiomyocytes combine to increase the risk of arrhythmias, with βAR stimulation being the necessary factor to reveal the CPVT1 phenotype in *RyR2*-R2474S. These data supports that the mainstay of treatment for CPVT1 is antagonism of βAR stimulation in ventricular cardiomyocytes.

Supporting information

S1 Fig. Western blots. Complete western blots with molecular size markers used in Fig 5. Blue arrows indicate analysed band. (A) GAPDH (goat polyclonal antibody), (B) CaMKII (rabbit polyclonal antibody), (C) pCaMKII (rabbit polyclonal antibody), (D) RyR (mouse monoclonal antibody), (E) pRyR Ser2808 (rabbit polyclonal antibody), (F) pRyR Ser2814 (rabbit polyclonal antibody), (G) PLB (mouse monoclonal antibody), (H) pPLB Ser16 (rabbit polyclonal antibody), (I) pPLB Thr17 (rabbit polyclonal antibody) and (J) SERCA2a (mouse monoclonal antibody).
(PDF)

S2 Fig. Computational model. The effects of increased pacing frequency and stimulation of βAR on RyR-RS and WT observed in cellular experiments were reproduced in a computational model of mouse ventricular myocyte electrophysiology and ion homeostasis. The only adaptation needed to recapitulate the difference between RyR-RS and WT was increased luminal RyR2 Ca2+ sensitivity by 10% in RyR-RS. (A) The left panel shows modelled representation of whole-cell intracellular Ca^{2+} ($[Ca^{2+}]i$) and SR Ca^{2+} content ($[Ca^{2+}]SR$) during the last three seconds of a pacing protocol similar to the one used in the cellular experiments, while the right panel shows the same parameters in a 10 s post-pacing period after cessation of pacing. (B) Bar graphs of results from the post-pacing period: The left panel shows the frequency of Ca^{2+} waves in a 10 s period after 0.5 and 4 Hz pacing in presence and absence of ISO. Increased pacing frequency increased the frequency of Ca^{2+} waves in the post-pacing period in both RyR2-RS and WT, while ISO increased the Ca^{2+} wave frequency more in RyR2-RS. The right panel shows the time to occurrence of the first Ca^{2+} wave after cessation of pacing, i.e. Ca^{2+} wave latency. Increased pacing frequency decreased Ca^{2+} wave latency in a post-pacing period in both RyR-RS and WT, while ISO decreased the Ca^{2+} wave latency more in RyR2-RS.F.
(PDF)

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III

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Exercise Training Stabilizes RyR2-Dependent Ca^{2+} Release in Post-infarction Heart Failure

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Aim: Dysfunction of the cardiac ryanodine receptor (RyR2) is an almost ubiquitous finding in animal models of heart failure (HF) and results in abnormal Ca^{2+} release in cardiomyocytes that contributes to contractile impairment and arrhythmias. We tested whether exercise training (ET), as recommended by current guidelines, had the potential to stabilize RyR2-dependent Ca^{2+} release in rats with post-myocardial infarction HF.

Materials and Methods: We subjected male Wistar rats to left coronary artery ligation or sham operations. After 1 week, animals were characterized by echocardiography and randomized to high-intensity interval ET on treadmills or to sedentary behavior (SED). Running speed was adjusted based on a weekly VO_{2max} test. We repeated echocardiography after 5 weeks of ET and harvested left ventricular cardiomyocytes for analysis of RyR2-dependent systolic and spontaneous Ca^{2+} release. Phosphoproteins were analyzed by Western blotting, and beta-adrenoceptor density was quantified by radioligand binding.

Results: ET increased VO_{2max} in HF-ET rats to 127% of HF-SED ($P < 0.05$). This coincided with attenuated spontaneous SR Ca^{2+} release in left ventricular cardiomyocytes from HF-ET but also reduced Ca^{2+} transient amplitude and slowed Ca^{2+} reuptake during adrenoceptor activation. However, ventricular diameter and fractional shortening were unaffected by ET. Analysis of Ca^{2+} homeostasis and major proteins involved in the regulation of SR Ca^{2+} release and reuptake could not explain the attenuated spontaneous SR Ca^{2+} release or reduced Ca^{2+} transient amplitude. Importantly, measurements of beta-adrenoceptors showed a normalization of $beta_1$-adrenoceptor density and $beta_1$:$beta_2$-adrenoceptor ratio in HF-ET.

Conclusion: ET increased aerobic capacity in post-myocardial infarction HF rats and stabilized RyR2-dependent Ca^{2+} release. Our data show that these effects of ET can be gained without major alterations in SR Ca^{2+} regulatory proteins and indicate that future studies should include upstream parts of the sympathetic signaling pathway.

Keywords: exercise training, arrhythmias, cardiac ryanodine receptor, heart failure, myocardial infarction

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INTRODUCTION

Current guidelines recommend exercise training (ET) as part of rehabilitation programs after myocardial infarction (MI) and for patients with heart failure (HF) (1, 2). ET has a range of beneficial cardiovascular effects (3) including improved aerobic capacity and cardiac contractile function (4–6) and decreases mortality after MI and in patients with HF (7, 8). However, approximately half of all patients with HF still die of ventricular arrhythmias (9). To exploit fully the therapeutic potential of ET in HF, we need a better understanding of its effects on the mechanisms for arrhythmias in HF.

The mechanism that underlies increased risk of arrhythmias in post-MI HF is multifactorial and includes fibrosis, altered expression or function of ion channels, and perturbed Ca^{2+} homeostasis (10). An almost ubiquitous finding in animal models of post-MI HF is a dysfunction of the cardiac ryanodine receptor (RyR2), i.e., the Ca^{2+} release channel of the sarcoplasmic reticulum (SR) (11, 12). This channel is essential in normal cellular Ca^{2+} handling and excitation–contraction coupling (13). RyR2 dysfunction can lead to diastolic Ca^{2+} leak from the SR, which contributes to contractile impairment and arrhythmias (14). Inhibition of SR Ca^{2+} leak has been found to prevent arrhythmias in animal models of HF, as well as arrhythmogenic events in cardiac tissue taken from patients with HF, and is a potential future therapeutic strategy (15, 16). However, no drugs that specifically target RyR2 are clinically available. Based on results from mouse models that show RyR2 dysfunction, ET might have the potential to stabilize RyR2 function and prevent arrhythmias (17, 18). However, there is a scarcity of data that support such an effect of ET in post-MI HF, and therefore, there is a need for rigorous experimental data from clinically relevant models.

MATERIALS AND METHODS

Ethical Approval

This investigation was approved by the Norwegian National Committee for Animal Welfare under the Norwegian Animal Welfare Act (FOTS ID: 4173 and 6577). It conformed to the National Institutes of Health guidelines (NIH Publication No. 85-23, revised 1996, US).

Animal Model of Post-infarction Heart Failure

A total of 52 male Wistar rats were included in the study. They either underwent sham operations or were subjected to MI by left coronary artery ligation. Left coronary artery ligation was performed through a thoracotomy under general anesthesia, which was achieved by inhalation of 65% N_2O, 32% O_2, and 2.5% isoflurane through an endotracheal tube, as previously described (19). Through a 2-cm incision in the skin over the sternum, the cutis on the left thorax was loosened from the underlying layer, and a left thoracotomy was performed in the fourth intercostal space. The pericardial sac was opened, and the left coronary artery was ligated ∼1 mm beneath the left atrium. In the control (sham) rats, the same operating procedure was employed, but the left coronary artery ligation was not performed. The skin was closed by sutures. Buprenorphine 0.2 mg/kg was administered subcutaneously for analgesia.

Rats with MI were evaluated after 1 week by 2D M-mode echocardiography. Based on previously established criteria, rats that exhibited a left atrial diameter of >5.0 mm were included in the HF group (15, 17). Rats within the same group (sham or HF) were paired according to weight and were randomly assigned either to a 5-week high-intensity ET program or to a sedentary, i.e. non-exercising, control group. The rats were housed together in cages under a 12:12 h light:dark cycle, with free access to water and food.

Protocol for Exercise Training

ET was initiated 1 week after coronary artery ligation. Separate treadmill and metabolic chambers were used for exercise training and for weekly measurements of maximal oxygen uptake (VO_{2max}) (Columbus Instruments, OH, USA), respectively. Three days before the first VO_{2max}-test, rats were habituated to the treadmill with daily 15-min exercise bouts at walking pace.

High-intensity exercise training has been shown to increase aerobic capacity in rats more than moderate exercise training (6). Pilot data, as well as previous publications, showed that the effect on VO_{2max} reached a plateau at 5 weeks (6, 20). Therefore, all ET rats completed a 5-week high-intensity ET protocol of treadmill running. Each training session lasted for 1 h. It consisted of a 10-min warm-up period and five 8-min intervals at 80–90% of the running speed that had been found to produce VO_{2max}, interspersed by 2-min rest periods at 60% running speed. The treadmill was set at an inclination of 25° at all times. The running speed was individualized for all rats according to a weekly VO_{2max}-test to keep the intensity constant throughout the ET program. SED rats ran on the treadmill at 60% of the speed that was found to produce VO_{2max} for 15 min, 2 days per week. All rats in the ET groups continued daily exercise until 1 day before euthanasia.

Echocardiography

Echocardiographic parameters were recorded with a Vevo2100 System (Fujifilm VisualSonics Inc., Canada) in rats that were anesthetized by a mixture of 65% N_2O, 33% O_2, and 2% isoflurane by mask ventilation. Echocardiography was performed 1 week after coronary ligation to enable stratification, and it was repeated at completion of the ET program (21).

Cardiomyocyte Calcium Imaging

Ventricular cardiomyocytes were isolated using constant-pressure perfusion of the coronary arteries with collagenase-containing solution (solution containing in mM 130 NaCl, 25 HEPES, 22 D-glucose, 5.4 KCl, 0.5 $MgCl_2$, 0.4 NaH_2PO_4, adjusted to pH 7.4 with NaOH), as previously described (22). The hearts were mounted on a Langendorff setup and were perfused retrogradely through the aorta with a 37°C solution that contained 0.08 mM Ca^{2+} and 200 U/ml collagenase type 2 (Worthington Biochemical Corporation, Lakewood, NJ, USA). The hearts were perfused for 20 min before the left ventricular tissue was excised rapidly, and the infarcted area was removed.

The ventricular tissue was then cut into small pieces and was gently mixed with a cutoff Pasteur pipette for about 1 min in a buffer that contained 1% bovine serum albumin (BSA) (Sigma Aldrich) and 0.02 U/ml deoxyribonuclease 1 (Worthington Biochemical Corporation, Lakewood, NJ, USA). The solution with the ventricular tissue was then filtered through a nylon mesh (pore diameter, 200 μm). After sedimentation, the cells were resuspended three times in 1% BSA solutions with increasing Ca^{2+} concentration (0.1, 0.2, and 0.5 mM, respectively). The cells were used for experiments within 10 h.

Whole-cell Ca^{2+} imaging was performed with a Zeiss Axiovert 200M microscope (Carl Zeiss Microscopy, LCC, NY, USA). Left-ventricular cardiomyocytes were field stimulated at 1, 2, and 4 Hz by a 3-ms symmetrical bipolar pulse, which was ∼20% above the voltage threshold for contraction of the individual cardiomyocytes. The experimental superfusate was based on modified HEPES–Tyrode's solution, and contained (in mM) HEPES 5, NaCl 140, KCl 5.4, $MgCl_2$ 0.5, glucose 5.5, NaH_2PO_4 0.4, and $CaCl_2$ 1. pH was adjusted to 7.4 with NaOH. Cytosolic Ca^{2+} was visualized the by use of 5 μM fluo-4 acetomethyl (AM) ester (Molecular Probes, Eugene, Oregon, USA), with 10-min loading before experiments. All experiments were performed at 37°C. For beta-adrenoceptor activation, 10 nM isoprenaline sulfate (ISO) (NAF, Norway) was added to the modified HEPES–Tyrode's solution. After addition of ISO, the Ca^{2+} transient amplitude reached steady state after ∼1 min. The cardiomyocytes were stimulated for another 30 s before measurements were made of the Ca^{2+} transients. Ca^{2+} waves were measured in a 10-s pause after the stimulation period. SR Ca^{2+} removal rate was estimated as the average rate of decay ($k = 1/\text{tau}$) that was taken from the last three Ca^{2+} transients before a pause. SR Ca^{2+} content was measured as peak fluorescence after rapid application of 10 mM caffeine at 1 Hz in the presence and absence of ISO. SERCA2-dependent Ca^{2+} removal was measured as the difference between the decay rate of Ca^{2+} transients and the decay rate of SR Ca^{2+} release after exposure to 10 mM caffeine. Ca^{2+} removal rate by the sodium–calcium exchanger (NCX) and plasma membrane Ca^{2+} ATPase (PMCA) was measured as the decay rate of the caffeine-induced Ca^{2+} release.

The relative increase in diastolic Ca^{2+} during pacing was measured as the difference in mean F_0 between 1, 2, and 4 Hz for three Ca^{2+} transients after 30 s stimulation.

Fractional release was measured as mean F of 3 Ca^{2+} transients after 30 s stimulation divided with F for caffeine release.

Confocal microscopy line-scan imaging was performed using a Zeiss LSM 7 Live confocal microscope (Carl Zeiss Microscopy, LCC, NY, USA). Cardiomyocytes were stimulated at 1 and 4 Hz in a protocol that was similar to the one described for whole-cell Ca^{2+} imaging measurements of Ca^{2+} waves, but with a 6-s post-stimulation rest period in which Ca^{2+} sparks were recorded.

Beta-Adrenoceptor Quantification by Radioligand Binding

To assess beta-adrenoceptor density, a radioligand binding assay was performed on left ventricles snap frozen in liquid nitrogen. Crude cell membrane fractions were prepared as described by Krobert et al. (23). Radioligand binding was performed as described by Ramberg et al. (24), where membranes were incubated with [^{125}I]-(−)iodocyanopincolol (0.066 nM) and the indicated concentration of either CGP20712A or ICI118551 for 2 h at 23°C. Data were fitted to a two-site binding model, and high and low binding affinities (pK_i) were calculated in GraphPad Prism 8.0.1 using a K_d of 0.04 nM (affinity of [^{125}I]-(−)iodocyanopincolol was determined in the left ventricular membranes). $Beta_1$-adrenoceptor density was determined as an average of high-affinity CGP20712A and low-affinity ICI118551 binding in the same heart. Similarly, $beta_2$-adrenoceptor density was determined as an average of high-affinity ICI118551 and low-affinity CGP20712A binding in the same heart.

Phosphoprotein Analysis

Western blots were performed on total protein homogenates from the left ventricles that had been stored at −70°C. Homogenates were denatured at 100°C for 5 min or at 37°C for 10 min in a sample buffer that contained 50% sucrose, 7.5% sodium dodecyl sulfate (SDS), 62.5 mM Tris–HCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.2 M dithiothreitol (DTT), and 0.01% bromophenol blue. Proteins were then fractionated according to size on 4–15% Criterion TGX gels (26 wells, 15 μl, Cat no. 567-1085, Bio-Rad Laboratories, Oslo, Norway) and blotted on 0.45 μM polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Oslo, Norway). The examination of phospholamban (Plb) was performed through use of 18% Criterion TGX gels (26 wells, 15 μl, Cat no. 567-1075, Bio-Rad Laboratories, Oslo, Norway). Membranes were blocked in 5% non-fat milk in Tris-buffered saline with 0.1% polysorbate 20 for 1 h at room temperature. Then, they were incubated with primary antibody overnight at 4°C and with secondary antibody for 1 h at room temperature. Blots were developed by application of enhanced chemiluminescence (ECL prime, GE Healthcare, Oslo, Norway), and signals were quantified using ImageQuant software (GE Healthcare, Oslo, Norway).

The primary antibodies for protein detection were anti-RYR2 phosphoserine-2814 (A010-31, Badrilla, Leeds, UK), anti-RYR2 phosphoserine-2808 (A010-30, Badrilla, Leeds, UK), ryanodine receptor (MA3-916, Thermo Fisher Scientific Inc., Rockford, IL, USA), anti-CaMKII (phospho-threonine286) (ab32678, Abcam PLC, Cambridge, UK), CaMKIIδ (22), anti-phospholamban phosphoserine-16, (A010-12, Badrilla, Leeds, UK), anti-phospholamban phospho-threonine-17, (A010-13, Badrilla, Leeds, UK), and anti-SERCA2a, Cat no. MA3-919 (Thermo Fisher Scientific Inc., Rockford, IL, USA), $beta_1$ adrenergic receptor antibody (PA1-049, Thermo Fisher), and monoclonal antivinculin antibody produced in mouse (V9131, Sigma-Aldrich). Secondary antibodies were antirabbit or antimouse immunoglobulin G (IgG) horseradish peroxidase (HRP)-linked whole antibodies, Cat no. NA934/NA931 (GE Healthcare, Oslo, Norway), diluted in the ratio 1:5,000. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (v-18, sc-20357, Santa Cruz Biotechnology Inc., CA, USA) was used as the loading control for all Western blots, except for phosholamban, and for $beta_1$ adrenergic receptor, for which vinculin was used. The membrane was reprobed with the specified antibodies with stripping of the membrane between each antibody when GAPDH was used as the loading control. HeLa Whole-cell lysate (sc-2200,

Santa-Cruz) was used as a positive control for Western blots of $beta_1$-adrenoceptor.

Statistical Analysis

All experiments and analyses were performed by investigators who were blinded to the phenotype and group identity of each cell and animal. Statistical tests were selected based on advice from an external statistical advisor. A paired Student's *t*-test was used to compare the effect of exercise training on VO_{2max} at baseline and after 5 weeks within each group. Unpaired nested ANOVA was used to compare ET and SED for all results from whole-cell Ca^{2+} imaging. In addition, two-way ANOVA was used to compare difference between ET and SED in the presence or absence of ISO at all frequencies for Ca^{2+} waves, Ca^{2+} transient amplitude, and Ca^{2+} decay rate. A unpaird Student's t-test was used to compare differences in Ca^{2+} waves frequency across frequencies and in absense and presence of ISO for HF cardiomyocytes. Ca^{2+} spark frequency was analyzed by application of a Poisson test to adjust for skewed distribution. One-way ANOVA was used when comparing beta-adrenoceptors. Bonferroni, Holm–Sidak, or Tukey's correction was performed when appropriate. Normal distribution was assessed with Shapiro–Wilk test. All statistics were performed by using IBM SPSS statistics 27, Sigmaplot 12.5 or R software (version 3.0.2, The R Foundation for Statistical Computing). $P < 0.05$ was considered statistically significant. Results are reported as the mean ± standard error of mean (SEM).

RESULTS

Heart Failure Model, Exercise Training, and Aerobic Capacity

We performed left coronary artery ligation on 23 male Wistar rats to induce large left ventricular myocardial infarctions. Sham operations were performed on 29 rats. One week after surgery, all rats were examined by echocardiography before randomization to ET or standard sedentary conditions (SED) (**Figure 1A**).

One week after surgery, the aerobic capacity of HF-ET rats was 86% of that of the sham-ET rats ($P < 0.05$) but did not differ from that of the HF-SED rats (**Figure 1B**). During the ET protocol, VO_{2max} increased in both ET groups, as expected. After 5 weeks of ET, VO_{2max} in the HF-ET group was 127% of that of the HF-SED group ($P < 0.05$), while VO_{2max} in the sham-ET group was 119% of that of the sham-SED group ($P < 0.05$, **Figure 1B**). However, VO_{2max} in the HF-ET group was only 88% of that of the sham-ET group (**Figure 1B**, $P < 0.05$).

Echocardiography was performed before randomization and repeated after completion of the ET protocol (**Figure 1C**). As per definition, the left atrial diameter was larger in the HF groups than in the sham groups at the time of randomization 1 week after surgery and had increased further in both HF groups when examined at week 6 ($P < 0.05$, **Figure 1D**). One week after surgery, HF rats also showed a clear phenotype with increased left ventricular diameter and reduced fractional shortening ($P < 0.05$, **Figures 1E,F**). During 5 weeks of ET, the left ventricular diastolic diameter (**Figure 1E**) and fractional shortening remained unchanged in the HF-ET group (**Figure 1F**), and ET did not affect any of the echocardiographic parameters in sham-operated rats (**Figures 1D–F**).

Spontaneous Calcium Release in Cardiomyocytes From HF Rats

We isolated cardiomyocytes from the left ventricles of the HF-ET and HF-SED rats at week 6 for the analysis of RyR2-dependent spontaneous Ca^{2+} release. We first recorded cardiomyocyte-wide propagated Ca^{2+} release events, i.e. Ca^{2+} waves, after a train of electrical stimuli at increasing frequencies (**Figures 2A,B**). We performed this protocol in the absence and presence of ISO to simulate sympathetic activation with beta-adrenoceptor stimulation. Ca^{2+} wave frequency increased with increasing pacing frequency and during exposure to ISO (**Figure 2B**). Importantly, Ca^{2+} wave frequency during ISO was lower in HF-ET cardiomyocytes than in those of HF-SED rats at 1 Hz (**Figure 2B**) and when considered across all frequencies with two-way ANOVA (**Figure 2C**). This finding indicated that ET stabilized Ca^{2+} handling in HF.

We also measured local Ca^{2+} release events, i.e., Ca^{2+} sparks, by confocal microscopy after a train of electrical stimuli (**Figure 2D**). The frequency of Ca^{2+} sparks increased with stimulation frequency (from 1 to 4 Hz), both in the presence and absence of ISO ($P < 0.05$), and was clearly lower in the HF-ET than in the HF-SED cardiomyocytes (**Figure 2E**). This further indicated that ET stabilized SR Ca^{2+} handling in HF.

Systolic Ca^{2+} Release in Cardiomyocytes From HF Rats

If ET is to be used as an antiarrhythmic intervention, negative effects on systolic Ca^{2+} release responsible for contraction should not outweigh the positive effects of ET on RyR2-dependent spontaneous Ca^{2+} release associated with arrhythmias. Surprisingly, however, when we analyzed Ca^{2+} transients across different pacing frequencies, the amplitude was lower in HF-ET than HF-SED both in the absence and presence of ISO (**Figures 3A–D**). Ca^{2+} transient decay rate was also lower in HF-ET than HF-SED, but only during exposure to ISO (**Figures 3E,F**). To explain these findings, SR Ca^{2+} content and Ca^{2+} removal were analyzed during caffeine exposure (**Figure 3G**). However, no difference in SR Ca^{2+} content was observed between HF-ET and HF-SED (**Figure 3H**) nor could the slowed removal be attributed to SERCA2-dependent or sarcolemmal Ca^{2+} removal alone (**Figures 3I,J**). We also analyzed the increase in diastolic Ca^{2+} that is expected in response to increased pacing frequencies but found no difference between HF-ET and HF-SED (**Figure 3K**). Lastly, we calculated fractional SR Ca^{2+} release but only observed a small increase in HF-ET compared HF-SED during ISO, which cannot explain stabilized RyR2-dependent SR Ca^{2+} release (**Figure 3L**) (25).

Phosphoproteins in Left Ventricles From HF Rats

To explore other potential explanations for the stabilization of RyR2-dependent spontaneous Ca^{2+} release that was observed after ET, phosphoproteins levels were quantified in HF-ET and HF-SED (**Figure 4**). We focused on RyR2 and the

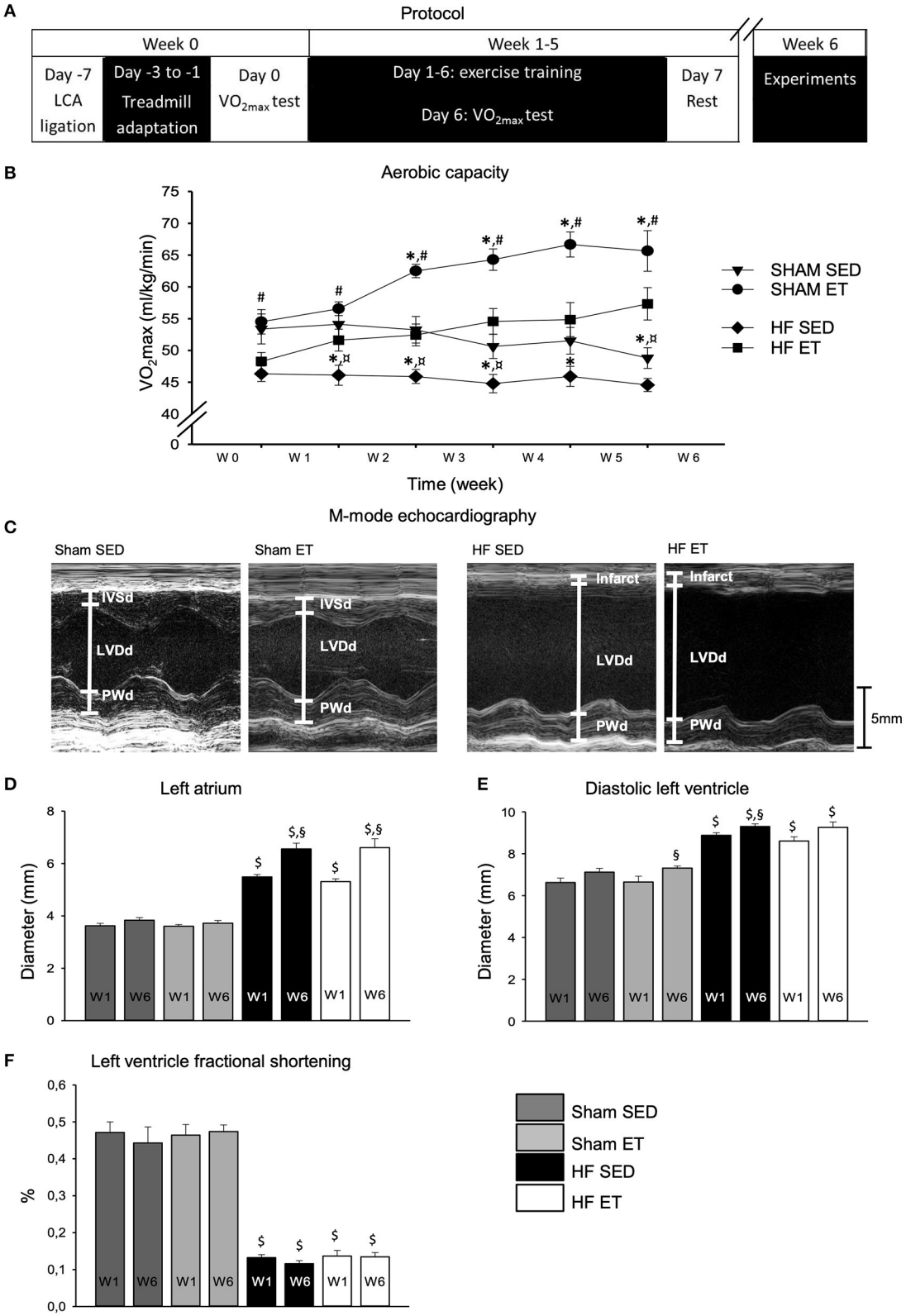

FIGURE 1 | Aerobic capacity and echocardiographic parameters. High-intensity exercise training improved aerobic capacity in sham-ET and HF-ET rats. **(A)** Exercise training protocol. **(B)** Mean VO_{2max} measurements from sham and heart failure (HF) rats. Number of rats in each group (SED/ET): sham 15/14, HF 13/10. Echocardiographic parameters were used for stratification of rats to the sham and HF groups. Representative M-mode images from the left ventricle in the parasternal

(Continued)

FIGURE 1 | long axis are shown for sham-SED **(C)** (left, n = 9), sham-ET (middle left, n = 8), HF-SED (middle right, n = 12), and HF-ET rats (left, n = 8). Bar graphs of the **(D)** left atrium diameter, **(E)** left ventricular diameter in diastole, and **(F)** left ventricle fractional shortening represent mean ± SEM. $P < 0.05$: $^{\$}$sham vs. HF; *SED vs. ET; $^{\#}$HF-ET vs. sham-ET; §week 1 vs. week 6; ¤HF SED vs. sham SED (Paired Student's *t*-test).

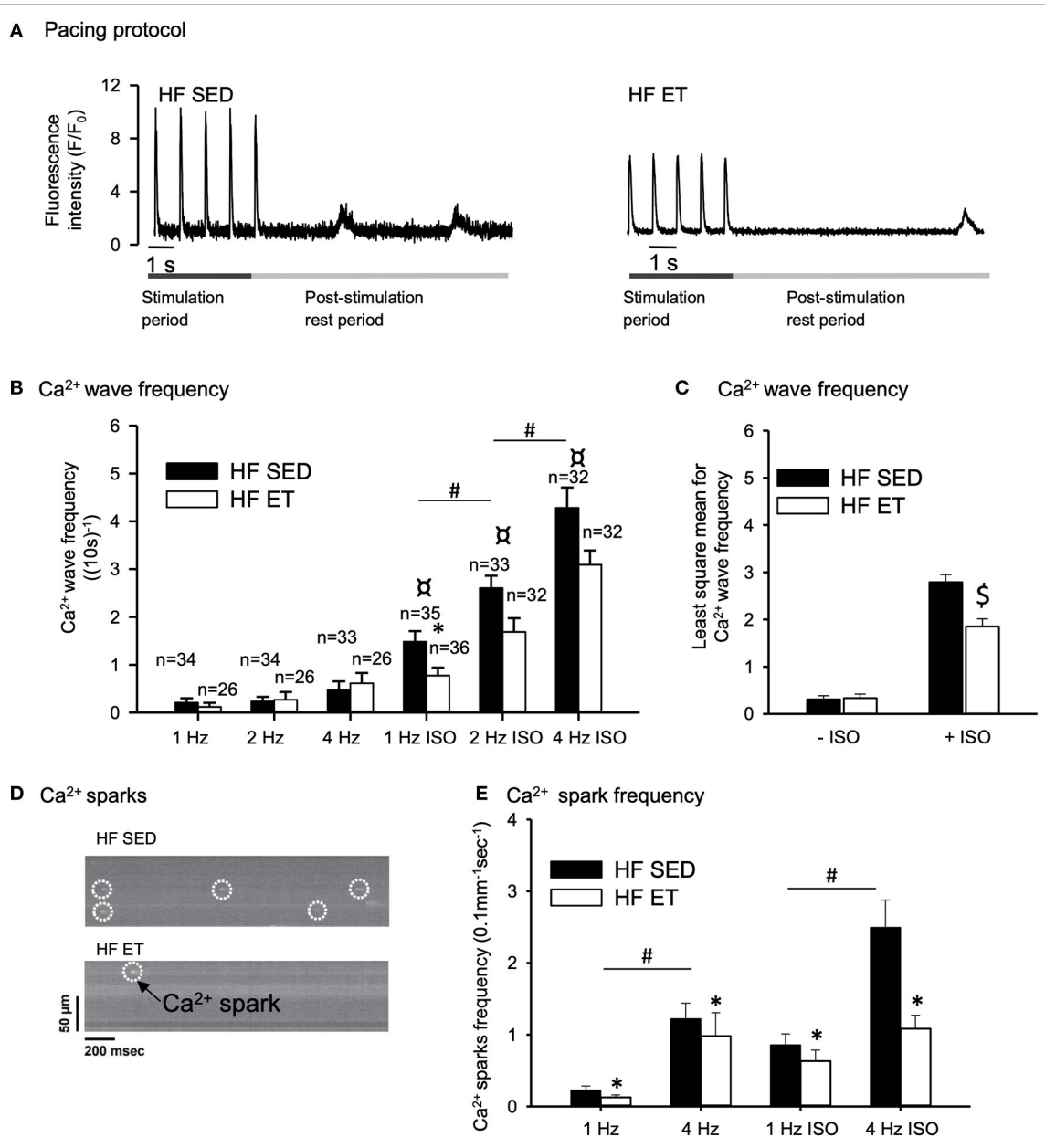

FIGURE 2 | Effect of exercise training on spontaneous Ca^{2+} release in heart failure (HF) cardiomyocytes. **(A)** Representative Ca^{2+} wave recordings and **(D)** confocal images of Ca^{2+} sparks are shown after field stimulation of HF-SED and HF-ET cardiomyocytes that were exposed to isoprenaline sulfate (ISO). Pacing at 1, 2, and 4 Hz was provided, and ISO was added to the experimental solution. Bar graphs of **(B)** Ca^{2+} wave frequency, **(C)** Ca^{2+} wave frequency across all pacing frequencies, and **(E)** Ca^{2+} spark frequency represent mean ± SEM. Number of rats in each group (SED/ET): Ca^{2+} wave frequency: $n = 6/5$, Ca^{2+} spark frequency: $n = 10/8$. Number of cells used for measurements of Ca^{2+} spark frequency (SED/ET): without ISO 101/81, with ISO 88/75. $P < 0.05$: *HF-SED vs. HF-ET (unpaired nested ANOVA); *(Poisson test for Ca^{2+} spark frequency, Bonferroni); $^{\#}$1 Hz vs. 2 and 4 Hz (Unpaired Student's *t*-test); ¤ +ISO vs. –ISO at same frequency (Unpaired Student's *t*-test), $^{\$}$least square mean for HF-SED vs. HF-ET (two-way ANOVA, Holm-Sidak).

SERCA/Plb system as the proteins that are mainly responsible for SR Ca^{2+} release and reuptake. However, no differences in these proteins or their major regulatory phosphorylation sites were observed (**Figures 4A–I**). The Plb/SERCA2a ratio was also unaltered in HF-ET compared with that in HF-SED ($P = 0.68$).

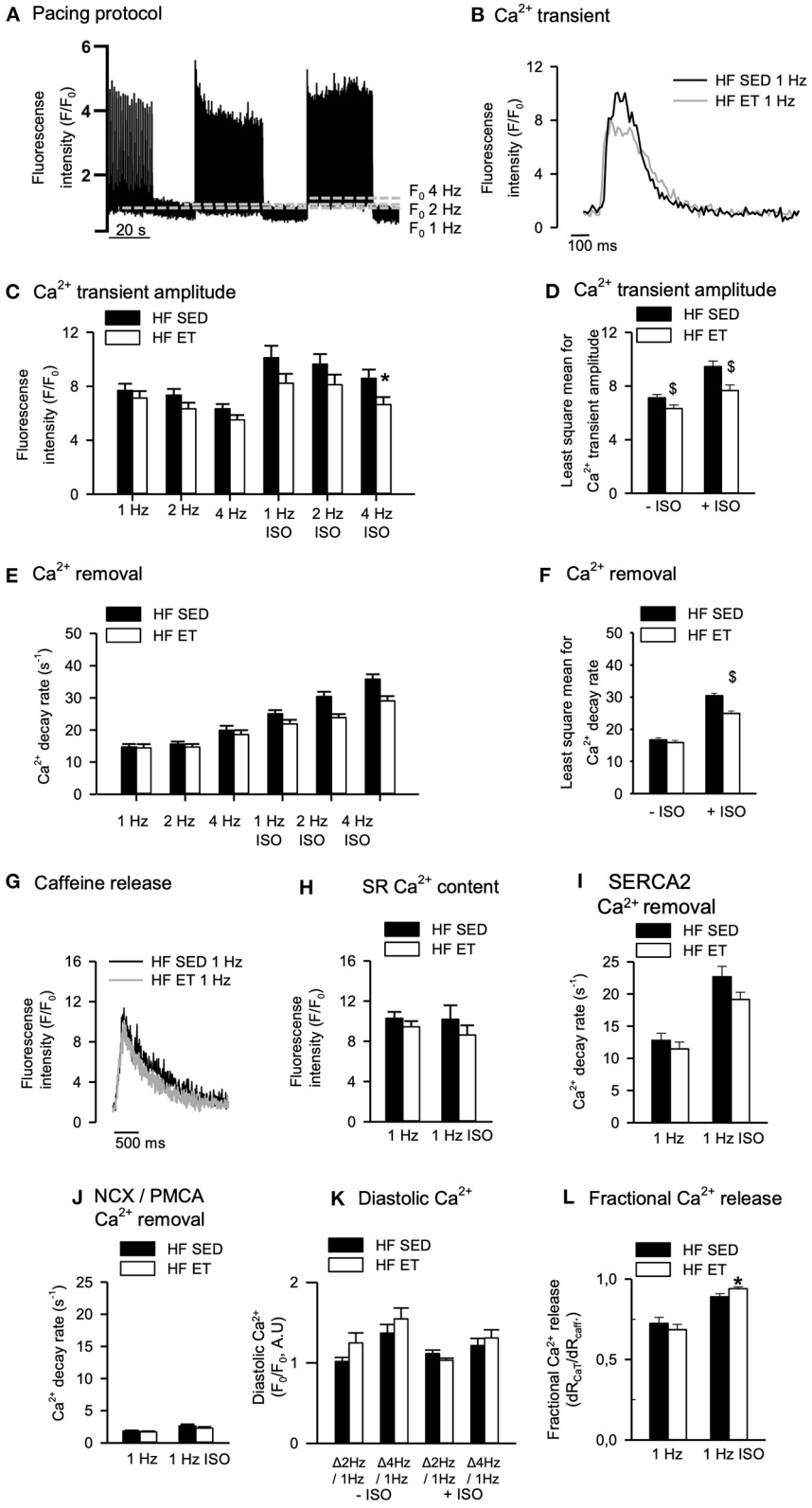

FIGURE 3 | Effects of exercise training on Ca^{2+} handling in heart failure (HF) cardiomyocytes. **(A)** Representative pacing protocol and **(B)** Ca^{2+} transients for HF-SED and HF-ET cardiomyocytes during 1 Hz stimulation and exposure to isoprenaline sulfate (ISO). **(C)** Ca^{2+} transient amplitude was reduced after exercise training at high stimulation frequency and ISO exposure and **(D)** across all frequencies with and without ISO exposure. **(E,F)** Ca^{2+} decay rate was lower across all frequencies with

(Continued)

FIGURE 3 | ISO exposure. **(G)** Representative tracings of SR Ca^{2+} content, measured by peak F/F_0 in response to a rapid application of 10 mM caffeine. Exercise training **(H)** did not affect SR Ca^{2+} content and **(I)** did not alter Ca^{2+} removal by SERCA2a or sodium–calcium exchanger (NCX) and plasma membrane Ca^{2+} ATPase (PMCA), as **(J)** measured by the decay rate of the caffeine-induced Ca^{2+} release. **(K)** Exercise training did not alter diastolic Ca^{2+}; **(L)** however, fractional release was higher in HF-ET compared to HF-SED when exposed to ISO. Number of rats (SED/ET): 6/5. Number of cells in **C–F**, **K** (SED/ET): –ISO 34/26, +ISO 35/36. Number of cells in **H–J**, **L** (ET/SED): HF 26/22, ISO 22/28. Bar graphs represent mean ± SEM. $P < 0.05$: *HF-SED vs. HF-ET (unpaired nested ANOVA), $^{\$}$least square mean for HF-SED vs. HF-ET (two-way ANOVA, Holm-Sidak).

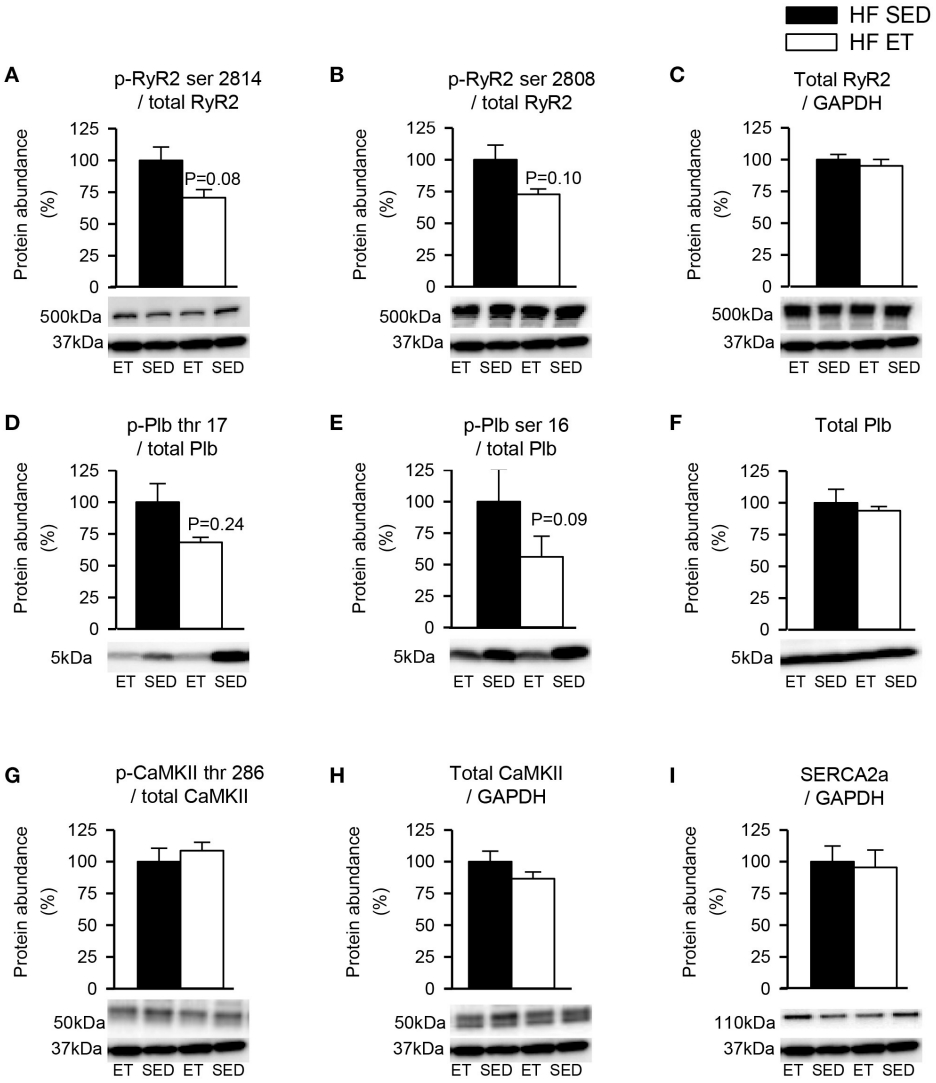

FIGURE 4 | Protein phosphorylation levels in heart failure (HF) left ventricles. Exercise did not decrease phosphorylation at sites that are known to be involved in destabilization of RyR2. Phosphoprotein levels were normalized to total protein levels. RyR2 phosphorylated at **(A)** serine 2814 (p-RyR2 ser 2814) and **(B)** serine 2808 (p-RyR2 ser 2808) were normalized to **(C)** total RyR2. Plb phosphorylated at **(D)** threonine 17 (p-Plb thr 17) and **(E)** serine 16 (p-Plb ser 16) were normalized to **(F)** total Plb and CaMKII phosphorylated at **(G)** threonine 286 (p-CaMKII thr 286) was normalized to **(H)** total CaMKII. **(I)** SERCA2a level was unaltered by exercise training. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control, except for Plb, and the membrane was reprobed with the specified antibodies with stripping of the membrane between each antibody, shown at 37 kDa. **(C,F,H,I)** Total protein levels were normalized to GAPDH levels. Number of rats (SED/ET): 6/5.

Effects of Exercise Training in Sham-Operated Rats

To control for untoward effects of ET, we performed most experiments in parallel in sham-ET and sham-SED rats (**Figures 5**, **6**). The ET protocol lowered Ca^{2+} wave frequency even in sham-ET compared to sham-SED both in the presence and absence of ISO when compared across all frequencies with two-way ANOVA (**Figures 5A–C**). However, Ca^{2+} spark frequency was similar in sham-ET and sham-SED (**Figures 5D,E**). ET had no effect on Ca^{2+} transients

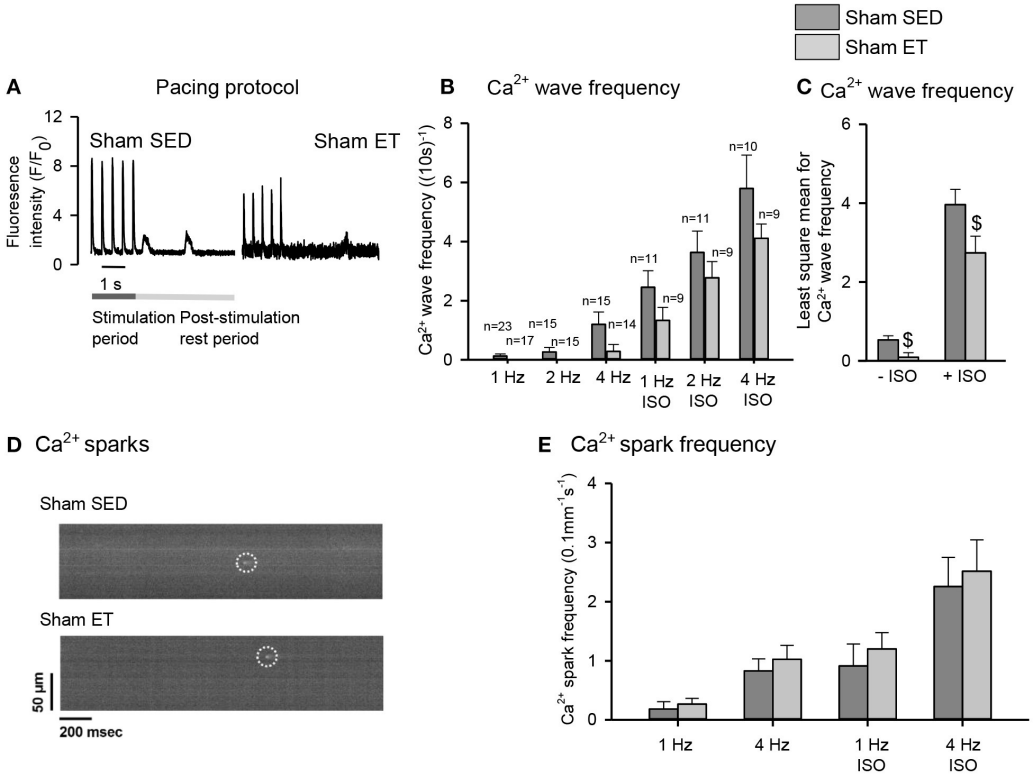

FIGURE 5 | Effect of exercise training on spontaneous Ca^{2+} release in sham cardiomyocytes. **(A)** Representative Ca^{2+} wave recordings, bar graphs of **(B)** Ca^{2+} wave frequencies at different pacing frequencies in the absence and presence of isoprenaline sulfate (ISO) and **(C)** two-way ANOVA comparing HF-ET with HF-SED. **(D)** Confocal images of Ca^{2+} sparks, and **(E)** bar graphs of Ca^{2+} spark frequency after different pacing frequencies. Number of rats (SED/ET): Ca^{2+} waves 4/3; Ca^{2+} sparks 7/6; number of cells (SED/ET): Ca^{2+} sparks: –ISO (60/53), +ISO (46/48); Ca^{2+} handling: –ISO (23/17), +ISO (10/9). $P < 0.05$: $^{\$}$least square mean for sham-SED vs. sham-ET (two-way ANOVA, Holm-Sidak).

amplitude (**Figures 6A–C**), Ca^{2+} removal rate, diastolic Ca^{2+} (**Figures 6D–F**), SR Ca^{2+} content (**Figures 6G,H**), or RyR2 phosphorylation (**Figures 6I,J**) in sham-operated animals.

Effects of Exercise Training on Beta-Adrenoceptors in HF and Sham-Operated Rats

As an alternative explanation for our observations, we examined if beta-adrenoceptor density was affected by exercise. For this, we used radioligand binding on membranes from left ventricular cardiomyocytes. The $beta_1$-adrenoceptor selective ligand CGP20712A displayed a pK_{i-high} of 9.21 ± 0.02 and a pK_{i-low} of 5.7 ± 0.03 (**Figure 7A**), while the $beta_2$-adrenoceptor selective ligand ICI118551 displayed a pK_{i-high} of 9.32 ± 0.04 and a pK_{i-low} of 6.9 ± 0.04 (**Figure 7B**), similar to previously reported values (26). The total beta-adrenoceptor density did not differ between the four groups (HF-ET, HF-SED, sham-ET, sham-SED, **Figure 7C**). Importantly, however, the $beta_1$-adrenoceptor density was significantly reduced in the HF-SED compared to sham-SED but increased back to normal levels in HF-ET (**Figures 7D,E**). The $beta_2$-adrenoceptor density, on the other hand, was increased in HF-SED compared to sham-SED and was not affected by ET (**Figure 7F**). In contrast to the effects of ET on membrane $beta_1$-adrenoceptor density, total abundance of the receptor measured by Western blotting was not altered by ET (**Supplementary Figure 1**).

DISCUSSION

We tested the hypothesis that ET could stabilize RyR2-dependent SR Ca^{2+} release associated with arrhythmias in post-MI HF rats. We subjected rats with HF to a 5-week ET protocol that was initiated 1 week after the induction of MI by ligation of the left coronary artery. This protocol increased the aerobic capacity of HF-ET rats to 127% of that of HF-SED rats but did not prevent a further increase in atrial diameter and had no impact on left ventricular diameter or on contractile function measured as fractional shortening. ET reduced the frequency of spontaneous Ca^{2+} release events in left ventricular cardiomyocytes, as indicated by a reduced frequency of Ca^{2+} waves and Ca^{2+} sparks in HF-ET cells compared with the HF-SED cells. In situations associated with increased adrenergic stress and beta-adrenoceptor stimulation, the HF-ET group exhibited lower Ca^{2+} transient amplitudes and decay rates than the HF-SED group, but no significant changes in RyR2, SERCA2 or their

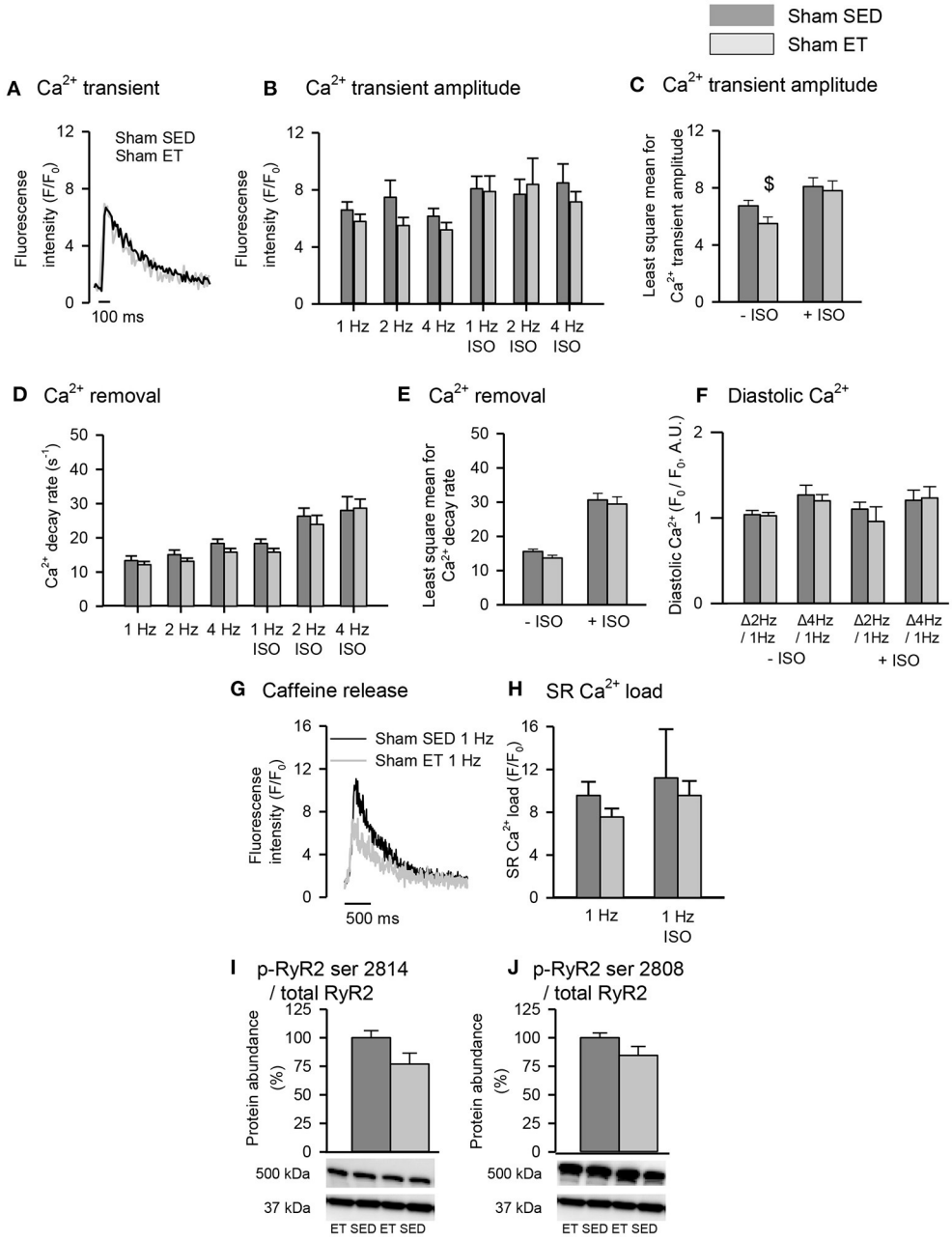

FIGURE 6 | Effect of exercise training on Ca^{2+} handling in sham cardiomyocytes. **(A)** Representative Ca^{2+} transient recordings is shown after field-stimulation of sham-SED and sham-ET cardiomyocytes. Stimulation of 1, 2, and 4 Hz was provided, and isoprenaline sulfate (ISO) was added to the experimental solution. Bar graphs of **(B,C)** Ca^{2+} transient amplitude, **(D,E)** Ca^{2+} removal and **(F)** diastolic Ca^{2+} represent mean ± SEM. **(G)** Representative tracings of SR Ca^{2+} content, measured by peak F/F_0 in response to a rapid application of 10 mM caffeine at 1 Hz. **(H)** Bar graphs of SR Ca^{2+} load, represents mean ± SEM. Phosphoprotein levels were normalized to total protein levels. RyR2 phosphorylated at **(I)** serine 2814 (p-RyR2 ser 2814) and **(J)** serine 2808 (p-RyR2 ser 2808). **(I,J)** Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control, and the membrane was reprobed with the specified antibodies with stripping of the membrane between each antibody, shown at 37 kDa. Number of rats (SED/ET): Ca^{2+} handling (4/3); proteins (5/5). Number of cells (SED/ET): Ca^{2+} handling: –ISO (23/17), +ISO (10/9). $P < 0.05$: $^{\$}$least square mean for sham-SED vs. sham-ET (two-way ANOVA, Holm-Sidak).

major regulatory proteins or phosphorylation sites. Sham-operated ET rats also exhibited a 135% increase in aerobic capacity compared to sham-SED and lower Ca^{2+} wave frequency when measured across all frequencies. However, we observed no changes in echocardiographic parameters or changes in Ca^{2+} measurements or SR Ca^{2+} regulatory proteins that can explain this. Since the Ca^{2+} regulatory proteins could not explain our main observation, we tested an alternative hypothesis and found that $beta_1$-adrenoceptors were downregulated in HF-SED but normalized with ET.

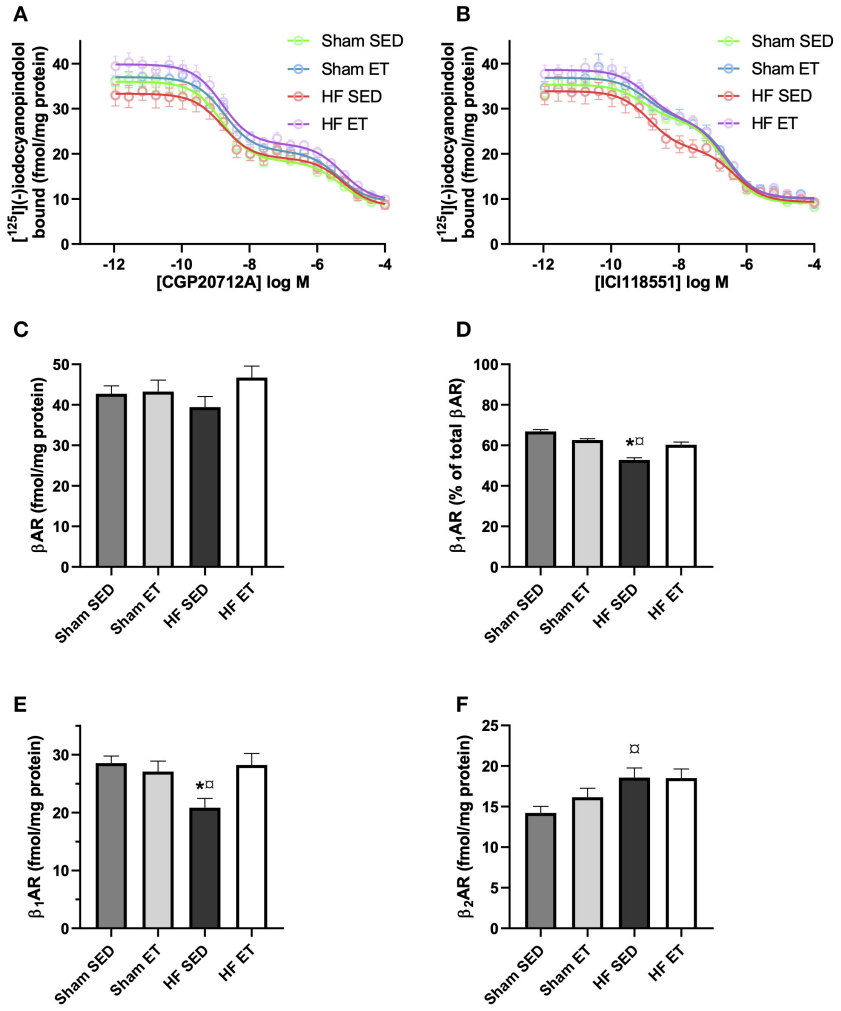

FIGURE 7 | Beta-adrenoceptor density in heart failure (HF) and sham left ventricles. Competitive binding assays between [^{125}I]-(–)iodocyanopindolol and the **(A)** beta$_1$-adrenoceptor selective ligand CGP20712A or the **(B)** beta$_2$-adrenoceptor selective ligand ICI118551 in membranes from the left ventricle from animals with the indicated treatment. The **(C)** total beta-adrenoceptor density and **(D)** percentage of beta$_1$-adrenoceptor was determined by a two-site binding model, where the average of high-affinity CGP20712A and low-affinity of ICI118551 is presented. **(E)** Beta$_1$-adrenoceptor density was determined as the average of high affinity of CGP20712ACFP20712A displacement and low affinity of ICI118551 displacement. **(F)** Beta$_2$-adrenoceptor density was determined as the average of high affinity of ICI118551 displacement and low affinity of CGP20712ACFP20712A displacement. The data shown are mean ± SEM of five (sham SED), four (sham ET), six (HF SED), and six (HF ET) animals. $P < 0.05$: *SED vs. ET, ¤HF SED vs. sham SED (one-way ANOVA, Tukey's correction).

Rat Model of Post-MI HF and RyR2 Dysfunction in Perspective

The pathophysiological changes that occur in post-MI HF are highly complex and progress over time (27). After a large MI, structural and mechanical alterations occur at the organ level, as well as in cardiomyocytes and the extracellular matrix (28). The progression of these alterations leads to different mechanisms being responsible for increased risk of arrhythmias at different time points after an MI (29). Altered Ca^{2+} handling due to perturbed expression and function of Ca^{2+} handling proteins is involved in arrhythmogenesis at all stages after MI, mainly because these alterations increase the risk of triggered activity (30, 31). Dysfunctional RyR2 is key in this arrhythmia mechanism and has been observed in several animal models of established post-MI HF (32–34). Our model is in line with other models of HF, with reduced aerobic capacity, severely dilated left ventricular diameters, and increased left-atrial diameters as a sign of long-standing increase in end-diastolic pressure, as well as reduced density of beta$_1$-adrenoceptors (35, 36). Many models of HF with a similar phenotype show post-translational modifications of RyR2, especially increased phosphorylation (12). Thus, dysfunction and modification of RyR2 have received much attention as potential targets for prevention of arrhythmias in HF (37). Our model is therefore highly relevant to test the hypothesis that ET can stabilize RyR2.

Exercise Training as Antiarrhythmic Therapy in Post-MI HF

There is widespread interest in HF pathophysiology, RyR2 dysfunction, and the beneficial effects of ET in HF. Therefore, it is a surprise that data on the effects of ET on RyR2 function in post-MI HF are scarce. Bonilla et al. subjected dogs with anterior MI to 10 weeks of ET and showed a highly reduced tendency toward ventricular fibrillation compared with sedentary dogs (38). In line with our study, they showed that the frequency of Ca^{2+} sparks in ventricular cardiomyocytes was reduced in dogs in the exercise group and that the abundance of RyR2 that was phosphorylated at serine 2814 was also reduced, compared with the sedentary group. However, there was no data that indicated that the dogs had HF. Kemi et al. performed a study of post-MI HF rats that showed many similarities with our study, although they employed a different rat strain (39). They too found a positive effect on Ca^{2+} wave frequency, but they did not analyze phosphoproteins. In other publications, the same group has provided compelling evidence that ET can have beneficial effects on Ca^{2+} handling and on T-tubule structure in normal rats (5, 17, 20, 40). Based on these and other data of disease models that show dysfunctional RyR2 (17, 18), we hypothesized that ET could have beneficial effects on RyR2 function. We focused specifically on serine 2808 and 2814 in RyR2, as there is mounting evidence that phosphorylation of these residues contributes to SR Ca^{2+} leak in HF and that prevention of such phosphorylation can prevent arrhythmogenic Ca^{2+} release (41, 42). It is therefore interesting that we observed beneficial effects on spontaneous SR Ca^{2+} release, without significant alterations in the abundance of key SR Ca^{2+} handling proteins, or CaMKII-or protein kinase A (PKA)-dependent phosphorylation. Although previous studies have clearly shown that ET can exert beneficial effects on SR Ca^{2+} handling through attenuated phosphorylation by CaMKII especially, our results show that other mechanisms may also contribute to a similar result. Interestingly, we observed that Ca^{2+} transient amplitude and reuptake of Ca^{2+} was lower in HF-ET rats during adrenergic stress. It was therefore somewhat surprising that this coincided with increased $beta_1$-adrenoceptor density compared to HF-SED. Our radioligand binding experiments show that the number of $beta_1$-adrenoceptors was reduced in HF rats and increased to normal levels by ET (**Figure 7**). Our experiments do not provide an explanation for how this is compatible with reduced reuptake or why the increased density was not reflected in the total abundance of the receptors when measured by Western blotting. We can only speculate that ET altered the relative pools of these receptors at the cell surface and receptors associated with the SR. Recent studies have shown that internalized and SR-bound $beta_1$-adrenoceptors exert an important regulatory role in SR Ca^{2+} release (43). These possible effects of ET should be explored further in future studies.

Limitations

We investigated Ca^{2+} handling after MI and ET and focused specifically on SR Ca^{2+} handling. Our studies lead us toward a role of beta-adrenoceptors in the effects of ET in HF rats, although the focus of the study did not allow us to conclude if this represents an explanation to reduced frequency of spontaneous Ca^{2+} release events after ET. Still, there is a risk that we missed a difference in phosphoprotein abundance after ET, due to low sample size (type II error) in our analysis, and such effects should therefore not be excluded. The reader should be aware that a one-sided test of CaMKII-dependent phosphorylation of RyR2 would have resulted in a $P < 0.05$. Based on previous data, the *a priori* assumption that ET would decrease (not increase) CaMKII activity could be claimed, thus supporting a one-sided test. Furthermore, our analyses of phosphoproteins were only performed in the absence of sympathetic stimulation. Analysis performed after f.ex. exposure to ISO could potentially have clarified some of the non-significant observations.

Another potentially contributing factor that should be included in future studies is altered cell ultrastructure. ET can effect cell size and has been shown to alter t-tubule organization (40). These aspects could be of importance but were not included in our study.

Our study is limited to cellular phenomena and does not include measurements of *in vivo* arrhythmias. This limits the potential for extrapolation to propensity for arrhythmias *in vivo*. However, other mechanisms are also important for antiarrhythmic effects of ET, such as alterations in repolarization reserve (38) or reductions in fibrosis levels (44), although the latter is debated after findings in healthy rats (45). As these other mechanisms were not the focus of our study, we would not have been able to ascertain a causal link between the cellular phenomena and arrhythmias regardless of any observed effects on arrhythmias.

Our model of HF is based on induction of large MIs, which leads to clear mechanical and structural alterations in the left ventricle at an early time point after the MI. Any extrapolation to HF that develops after smaller MIs, which involves less remodeling, is unclear. Especially, we did not separate cardiomyocytes according to relative localization or distance from the MI, although the effects of ET could vary between regions (46, 47).

We chose to start high-intensity ET at an early time point after MI. High-intensity ET was chosen based on previous studies in rats (6). Moderate-intensity ET might have been more in line with what patients can perform, although high-intensity interval training is feasible even in patients with severe HF (48). Any extrapolation to correspondingly large MIs in humans is speculative, and no conclusion regarding the time at which ET should be initiated in patients can be drawn from studies in rats. The early initiation that was chosen in our study could mean that remodeling processes were still highly active, which could have affected our results. However, it could be argued that this is a reason to stress early initiation of ET in order to affect beneficially the remodeling process. We did not compare effects of exercise in HF and sham rats for all data, as this was not the focus of our study. The effects of ET could be different in diseased and normal animals, and the therapeutic potential should be tested in relevant disease models.

CONCLUSIONS

ET can stabilize RyR2-dependent Ca^{2+} release in post-MI HF. Our data indicate that the protective mechanism involves

regulation of both SR Ca^{2+} release and reuptake, and that effects of ET at several levels of the sympathetic signaling pathways controlling SR Ca^{2+} release should be explored in future studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Norwegian National Committee for Animal Welfare.

AUTHOR CONTRIBUTIONS

TD, MS, RM, JA, MF, MH, KA, KH, and IS conceived and designed the analysis, collected data, contributed to data analysis, performed the analysis, and wrote the paper. FL, WL, OS, and MS conceived and designed the analysis, contributed to data analysis, and wrote the paper. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2020.623922/full#supplementary-material

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