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Review

Targeting ryanodine receptors for anti-arrhythmic therapy

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Antiarrhythmic drugs are a group of pharmaceuticals that suppress or prevent abnormal heart rhythms, which are often associated with substantial morbidity and mortality. Current antiarrhythmic drugs that typically target plasma membrane ion channels have limited clinical success and in some cases have been described as being pro-arrhythmic. However, recent studies suggest that pathological release of calcium (Ca^{2+}) from the sarcoplasmic reticulum via cardiac ryanodine receptors (RyR2) could represent a promising target for antiarrhythmic therapy. Diastolic SR Ca^{2+} release has been linked to arrhythmogenesis in both the inherited arrhythmia syndrome 'catecholaminergic polymorphic ventricular tachycardia' and acquired forms of heart disease (eg, atrial fibrillation, heart failure). Several classes of pharmaceuticals have been shown to reduce abnormal RyR2 activity and may confer protection against triggered arrhythmias through reduction of SR Ca^{2+} leak. In this review, we will evaluate the current pharmacological methods for stabilizing RyR2 and suggest treatment modalities based on current evidence of molecular mechanisms.

Introduction

The cardiac ryanodine receptor (RyR2) is a homotetrameric Ca^{2+} release channel located in the sarcoplasmic reticulum (SR) membrane[1, 2]. During the normal cardiac cycle, plasma membrane depolarization initiates opening of L-type Ca^{2+} channels (LTCC), by which extracellular Ca^{2+} enters the cytoplasm. Ca^{2+} influx acts as a trigger that subsequently activates RyR2 channels, leading to a ten-fold greater release of SR Ca^{2+} into the cytoplasm. During systolic contraction of the heart, elevated cytoplasmic Ca^{2+} binds to troponin-C, allowing actin and myosin to interdigitate and cause sarcomere shortening, thus causing myocardial contraction. Diastolic relaxation occurs when cytoplasmic [Ca^{2+}] decreases as Ca^{2+} is extruded through the Na^+/Ca^{2+}-exchanger (NCX) or is actively pumped back into the SR through the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2a)[3]. Concomitantly, myocardial relaxation is directly associated with diastolic reduction in Ca^{2+} levels. Thus, physiologic control of Ca^{2+} release from the SR is necessary for timely contraction and relaxation during the cardiac cycle. Pathological "leak" of Ca^{2+} during diastole may be detrimental

and lead to cardiac arrhythmias[4, 5].

There is now considerable evidence that abnormal RyR2-mediated Ca^{2+} release from the SR can lead to both atrial[6, 7] and ventricular arrhythmias associated with sudden cardiac death[8–10]. Increased SR Ca^{2+} release during diastole can lead to activation of the Na^+/Ca^{2+}-exchanger[11], which in turn generates a transient inward current that can cause afterdepolarizations and triggered action potentials. These afterdepolarizations have been observed in humans and have been directly linked to arrhythmogenesis in animal models of arrhythmias[12].

Genetic susceptibility to cardiac arrhythmias may arise directly from genetic mutations in RyR2, such as in patients with catecholaminergic polymorphic ventricular tachycardia (CPVT)[9, 13]. Mutations in other proteins that bind to the pore-forming subunits within the RyR2 macromolecular complex (eg, calsequestrin, junctophilin) also have been reported to confer genetic susceptibility to cardiac arrhythmias and/or cardiomyopathy[14, 15]. These observations provide direct evidence that a perturbation in RyR2 function can facilitate the development of cardiac arrhythmias.

Additionally, acquired structural heart disease, for example heart failure or myocardial ischemia, has been shown to modify the post-translational regulation of RyR2 through

nitrosylation, oxidation, and phosphorylation, which might also increase susceptibility to diastolic Ca^{2+} release and arrhythmias[16–20]. Given that there are many excellent reviews on strategies to modify intracellular signaling to reduce RyR2 activation[21, 22], we will restrict the scope of this review mainly to pharmacological strategies to stabilize RyR2 directly to reduce arrhythmic potential.

Because RyR2 also plays an important role during excitation-contraction coupling, it is important that antiarrhythmic compounds targeting the RyR2 channel complex will not interfere with systolic SR Ca^{2+} release. At the same time, inhibition of diastolic SR Ca^{2+} release is a desirable feature of compounds that could prevent arrhythmias[22]. RyR2 activity can be modulated by numerous natural and pharmacological compounds, as reviewed elsewhere in more detail[22–24]. These compounds may modulate RyR2 in various ways, including by modulating channel gating, ion channel translocation, RyR2 subunit composition, or posttranslational modifications. Some of these compounds have emerged as strong candidates for antiarrhythmic drugs, and will be discussed in more detail below.

Dantrolene

Dantrolene sodium is a hydrantoin derivative that was initially described as a muscle relaxant[25], but later found to be a potent therapeutic agent for patients suffering from the rare life-threatening condition known as malignant hyperthermia (MH)[26]. Patients susceptible to MH typically have inherited mutations in the type 1 ryanodine receptor (RyR1) primarily found in skeletal muscle[27]. Exposure to inhaled halogenated anesthetics during surgery can trigger massive RyR1-mediated Ca^{2+} release associated with muscle breakdown, elevation of serum creatinine kinase (CK), hypotension, hyperthermia, and tachycardia, which often results in intraoperative death [28]. Dantrolene has been shown to directly bind to the N-terminus of RyR1 and to prevent SR Ca^{2+} leak in skeletal muscle, thereby improving clinical outcomes[29]. Dantrolene is believed to stabilize interdomain interactions between the N-terminal and central domains of RyR1[30] and RyR2[31], although the effects of dantrole on single RyR channels remains controversial[32].

Given that dantrolene improves the stability of both RyR1 and RyR2, dantrolene has become a molecule of interest for preventing cardiac arrhythmias. Dantrolene has previously been described as an inhibitor of arrhythmias in animal models of ischemia-reperfusion[33–35]. More recently, dantrolene was demonstrated to inhibit catecholaminergic polymorphic ventricular tachycardia in a knock-in mouse model heterozygous for mutation R2474S in RyR2[36] (Figure 1). Dantrolene was shown to suppress isoproterenol-induced spontaneous SR Ca^{2+} releases (ie, sparks) in intact myocytes isolated from RyR2-R2474A/+ mice. The mechanisms by which dantrolene prevented CPVT was attributed to stabilization of mutant RyR2 channels, and possibly also by preventing the PKA-induced reduction in calmodulin binding to RyR2[37]. In this study, dantrolene did not exert any appreciable effects on cardiac function in hearts of wild-type mice. However, dan-

trolene did correct defective interdomain interactions within RyR2 isolated from dogs with heart failure, associated with suppression of delayed afterdepolarizations[31].

In other studies, dantrolene has been described to improve cardiac contractility in failing hearts, which may contribute to its role in reducing arrhythmias in structural heart disease. Congestive heart failure (CHF) has been associated with a negative force-frequency relationship (Bowditch effect) in failing myocardium. Dantrolene has been shown to ameliorate the negative force-frequency relationship in explanted failing myocardial muscle strips by improving inotropic response to isoproterenol[38]. This improved contractility was not associated with overall changes in cytoplasmic $[Ca^{2+}]$, and was postulated to be associated with improvement of diastolic Ca^{2+} release. Further evidence for reduction of Ca^{2+} release events in improved contractility was shown by Kobayashi *et al*[31], who examined the effects of dantrolene on cardiac function on failing hearts. Dantrolene was shown to directly bind near the N-terminal domain in RyR2 at amino acids 601–620, a site critical for N-terminal and central domain interactions. Whereas unzipping of N-terminal and central domains was associated with spontaneous SR Ca^{2+} leak, dantrolene suppressed both unzipping and SR Ca^{2+} leak (sparks) and ultimately delayed afterdepolarizations, which are common in heart failure. Additionally, dantrolene restores calmodulin (CaM) binding to RyR2, which is usually attenuated in heart failure[39]. Thus, dantrolene appears to be a promising molecule to treat arrhythmias in patients with CPVT and may attenuate cardiac Ca^{2+} handling dysfunction associated with heart failure.

1,4-Benzothiazepines

The benzothiazepine derivative JTV519 (4-[3(1-(4-benzyl) piperidinyl)propionyl]-7-methoxy-2,2,4,5-tetrahydro-1,4-benzothiazepine; also known as K201) was first identified as a compound able to suppress intracellular Ca^{2+} overload associated with cardiac cell death[40]. The drug has been reported to have antiarrhythmic effects in a canine model of atrial fibrillation due to sterile pericarditis[41] and Langendorff-perfused rat hearts subjected to ischemia-reperfusion[42, 43]. JTV519 interacts with annexin-V and at higher doses inhibits various voltage-gated ion channels in the heart[44, 45]. Subsequently, it has become clear that RyR2 represents an important target of JTV519[46, 47].

JTV519 was described to normalize RyR2 gating in dogs with tachycardia-induced heart failure[48]. In this study, Kohno *et al*[48] demonstrated that JTV519 reversed the SR Ca^{2+} release defects indicative of RyR2 dysfunction. The concept of RyR2 stabilization by FKBP12.6 was further supported by the findings that JTV519 treatment of dogs with pacing-induced heart failure increased the amount of FKBP12.6 immunoprecipitated with RyR2[46]. Moreover, JTV519 was shown to prevent lethal ventricular arrhythmias in mice haploinsufficient for FKBP12.6 by increasing FKBP12.6 binding to RyR2[47] (Figure 2). The lack of efficacy of JTV519 in FKBP12.6 deficient mice suggests that FKBP12.6 binding to RyR2 is associated with the therapeutic effects of this compound[47]. On the other hand,
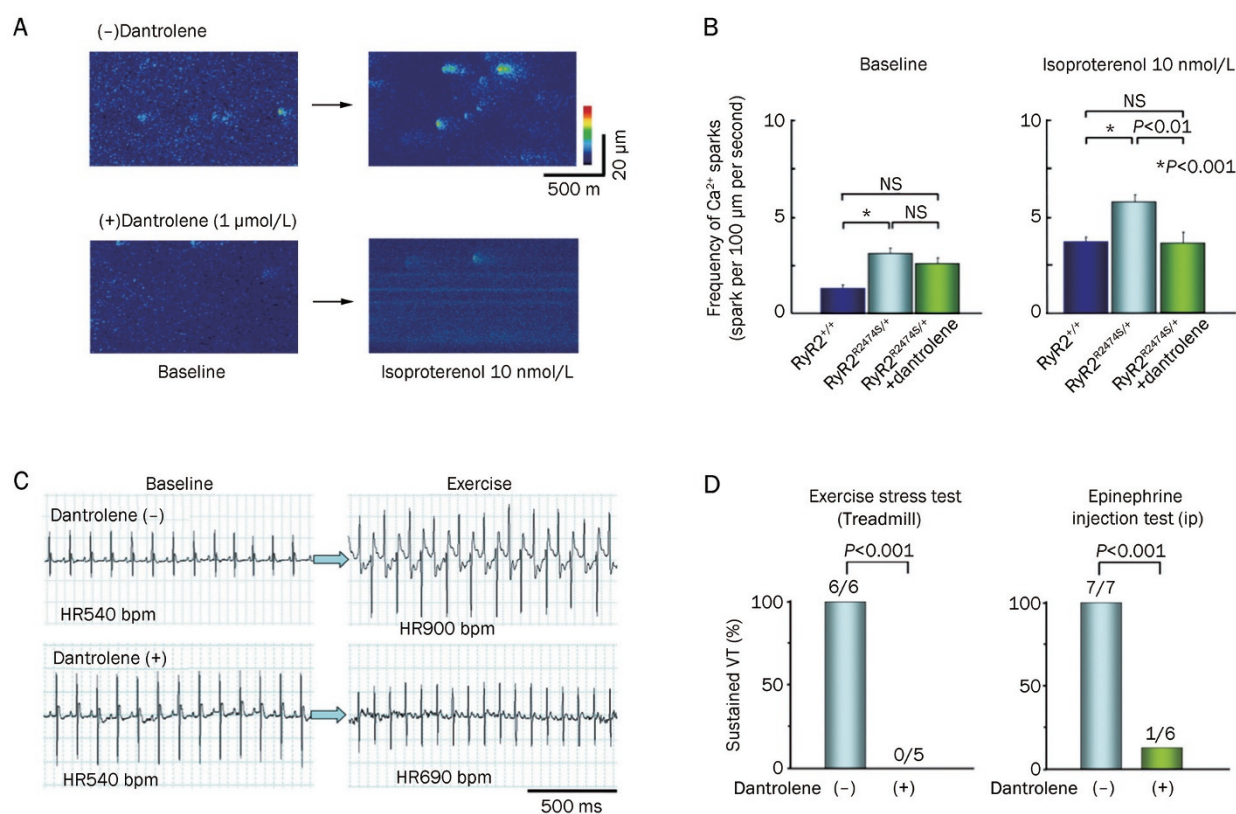
Figure 1. Dantrolene inhibits catecholaminergic polymorphic ventricular tachycardia in mice. (A) Representative images of Ca^{2+} sparks in cardio-myocytes isolated from heterozygous RyR2-R2474S/+ knock-in mice, showing that dantrolene reduces Ca^{2+} spark frequency following isoproterenol exposure. (B) Bar graph showing that dantrolene suppresses abnormal Ca^{2+} spark frequency in RyR2-R2474S/+ mutant mice after isoproterenol exposure. (C) Telemetric ECG recordings reveal exercise-induced ventricular tachycardia in a RyR2-R2474S/+ mouse, which was suppressed by dantrolene. (D) Bar graphs revealing that dantrolene suppresses the incidence of exercise or epinephrine induced ventricular tachycardia (VT) in RyR2-R2474S/+ mice. Adapted from Kobayashi *et al*[36].

normalizing FKBP12.6 levels within the RyR2 macromolecular complex stabilizes the closed state of the channel, thereby preventing aberrant openings during diastole[5]. Follow-up *in vitro* studies of human RyR2 mutations found in patients with CPVT (P2328S, Q4201R, and V4653F) showed that JTV519 can also normalize mutant channel gating as evidenced by single channel recordings[49].

There has been some controversy whether the antiarrhythmic effects of JTV519 require modification of RyR2-FKBP12.6 interactions[50]. In a mouse model of CPVT caused by the R4496C mutation in RyR2, it was shown that this mutation did not alter FKBP12.6 binding affinity for RyR2. Moreover, JTV519 did not prevent delayed afterdepolarizations in myocytes isolated from heterozygous RyR2-R4496C/+ mice[51]. However, subsequent studies by other groups have shown that JTV519 did reduce the occurrence of spontaneous action potentials in ouabain-treated WT and RyR2-R4496/+ mouse myocytes, presumably independent of FKBP12.6[52]. Additionally, *in vitro* studies in HEK293 cells suggest that JTV519 suppresses store-overload induced Ca^{2+} release independently of FKBP12.6 binding, though the relevance of these observations have yet to be determined *in vivo*[50]. Also, Yamamoto *et al*[53]

reported that JTV519 directly bound to RyR2 between amino acids 2114 and 2149, and that JTV519 can normalize defective interdomain interactions associated with RyR2 dysfunction.

Recently S107, a new 1,4-benzothiazepine similar to JTV519, has been found to prevent ventricular arrhythmias in a CPVT mouse model heterozygous for mutation R2474S in RyR2[54]. In contrast to JTV519, S107 has been reported to lack off-target activity for ion channels other than RyR2 at concentrations up to 10 mmol/L[54, 55]. S107 provided protection against epinephrine-induced ventricular tachycardias caused by abnormal SR Ca^{2+} leak in RyR2-R2474S/+ mice[54]. Further, S107 was recently shown to be effective at preventing ventricular arrhythmias in the *mdx* mouse model of muscular dystrophy[56]. Thus, 1,4-benzothiazepine derivatives JTV519 and S107 hold promise as RyR2-stabilizing molecules that could reduce the risk of arrhythmias[57].

Flecainide

Flecainide is a trifluoroethoxybenzamide that was discovered to be a potent antiarrhythmic agent in 1977[58]. Flecainide initially showed promise as an antiarrhythmic agent against both ventricular[59] and atrial arrhythmias[60]. Because a predomi-
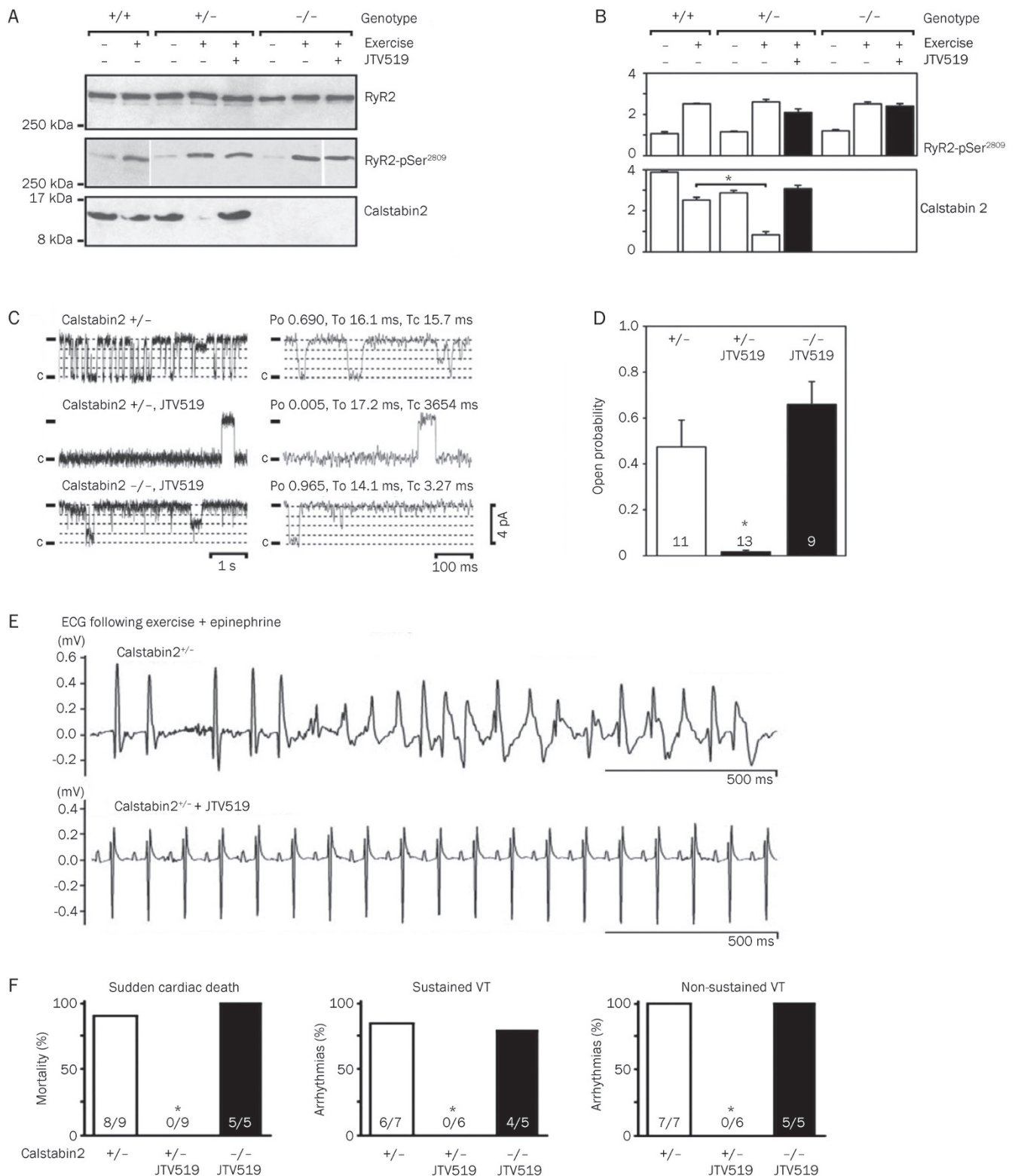
Figure 2. Anti-arrhythmic effects of 1,4-benzothiazepine JTV-519 in FKBP12.6[+/−] mice. (A–B) Representative immunoblots and quantifications for RyR2, RyR2-pSer[2809] (PKA phosphorylation site on RyR2), and calstabin2 (FKBP12.6) from wildtype (WT), calstabin2^{+/−} heterozygous, and calstabin2^{−/−} (FKBP12.6[−/−]) knockout mice. Whereas exercise increased PKA phosphorylation of RyR2 and decreased calstabin2 (FKBP12.6) binding to RyR2, JTV-519 prevented calstabin2 dissociation. (C–D) Representative single channel recordings in planar lipid bilayers showing that JVT519 reduced the open probability of RyR2 isolated from calstabin2^{+/−} but not calstabin2^{−/−} mice, consistent with calstabin2 (FKBP12.6) being required for the therapeutic effects of JTV519. (E–F) ECG tracings showing that JTV-519 reduces ventricular arrhythmias in calstabin2^{+/−} but not calstabin2^{−/−} mice. *P<0.05. Adapted from Wehrens *et al*[47].

nant mechanism of action on inactivation of voltage-gated Na$^+$ channels, it was classified as a type IC anti-arrhythmic drug. However, clinical trial results indicated that in patients with structural heart disease susceptible to ventricular arrhythmias, flecainide might in fact be pro-arrhythmogenic[61, 62].

Recently, there has been a resurgence of enthusiasm for the use of flecainide in a select group of CPVT patients with genetic predisposition to ventricular arrhythmias and SCD. Watanabe *et al*[63] found that flecainide inhibited the RyR2 channel by reducing the duration of RyR2 channel openings without affecting closed channel duration. Flecainide reduced SR Ca^{2+} release events and triggered arrhythmic beats in a calsequestrin deficient (*Casq2$^{-/-}$*) mouse model of CPVT [63] (Figure 3). Moreover, it was shown that flecainide significantly reduced the incidence of exercise-induced arrhythmias in patients with mutations in *CASQ2*[63]. Follow-up studies from the same group showed that flecainide reduced Ca^{2+} spark mass but increased spark frequency, resulting in a net neutral effect on SR Ca^{2+} leak and SR Ca^{2+} content[64]. This finding is distinct from the reported mechanism of tetracaine, another RyR2 channel blocking agent, which reduces Ca^{2+} sparks and SR leak, thereby increasing SR Ca^{2+} content. Therefore, it was concluded that flecainide promoted block of the RyR2 open state, reducing the "probability of saltatory wave propagation between adjacent Ca^{2+} release units"[64].

Other groups have applied these findings to further delineate the mechanism of arrhythmogenesis in another mouse model of CPVT. Knock-in mice heterozygous for mutation R4496/+ in RyR2 were crossed with *Cntn2-EGFP* transgenic mice expressing a fluorescent marker for the cardiac conduction system[65]. Whereas tetracaine reduced spontaneous SR Ca^{2+} release events in ventricular myocytes and Purkinje cells equally, flecainide more specifically targeted mutant RyR2 in Purkinje cells, implicating the Purkinje conduction system as a potent mediator of ventricular arrhythmias in CPVT. Thus, flecainide may have a unique role in the prevention and suppression of ventricular arrhythmias in patients with genetically inherited CPVT.

Modulation of RyR2 posttranslational modification

In addition to inherited mutations, RyR2 channel function may also be perturbed due to acquired changes in, for example, channel posttranslational modulation[2]. Xu *et al*[20] demonstrated that increased *S*-nitrosylation leads to enhanced RyR2 activity and promotes SR Ca^{2+} release. Increased *S*-nitrosylation of RyR2 has been associated with cardiac arrhythmias in a mouse model of Duchenne's muscular dystrophy, and inhibition with S107 (see above) was shown to normalize both RyR1 and RyR2 function and prevent arrhythmias[56, 66]. In contrast, Gonzalez *et al*[17] demonstrated that decreased rather
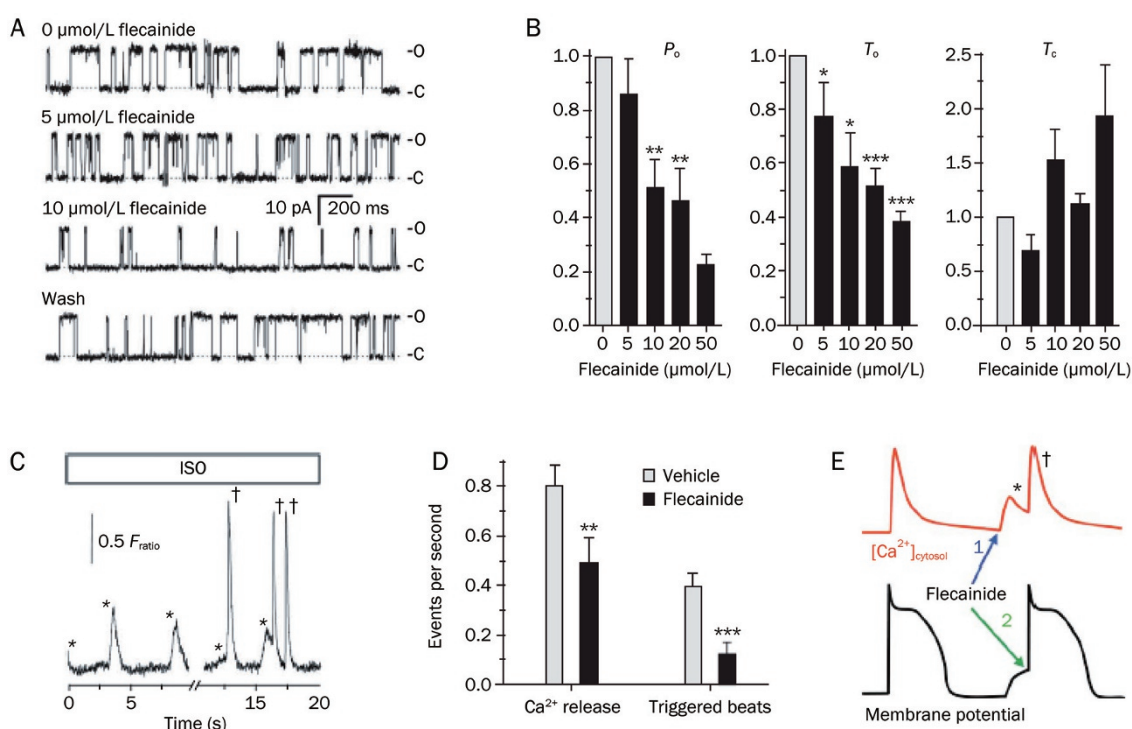

Figure 3. Prevention of triggered arrhythmias by flecainide. (A–B) Concentration-dependent effects of flecainide on single sheep RyR2 channels in lipid bilayers. Flecainide decreases open probability (Po) and mean open time (To), and does not significantly alter the mean closed time (Tc) of RyR2. *P<0.02, **P<0.01 and ***P<0.001. (C–D) Effects of flecainide on isoproterenol (ISO) stimulated calsequestrin-deficient cardiomyocytes. Whereas ISO evoked spontaneous SR Ca^{2+} release events (*), flecainide reduced the number of Ca^{2+} releases and triggered beats (†). **P=0.0078 and ***P <0.001. (E) Cartoon illustrating dual effects of flecainide action on SR Ca^{2+} release (red tracing) and inhibition of premature beats triggered by delayed after depolarization (black tracing). Adapted from Watanabe *et al*[63].

than increased *S*-nitrosylation of RyR2 might promote SR Ca^{2+} leak and arrhythmogenesis. One explanation of this apparent paradox relates to nitroso-redox imbance, a condition in which excess formation of reactive oxygen species (ROS) can modify the same thiols that are also target of *S*-nitrosylation[67]. Indeed, Gonzalez *et al*[67] reported evidence for increased oxidation of RyR2 associated with an increased tendency towards SR Ca^{2+} leak in rats with heart failure. In this particular study, increased oxidative stress was primarily the result of enhanced xanthine oxidase (XO) activity. Pharmacological inhibition of XO restored both the nitroso-redox imbalance and intracellular Ca^{2+} release defects in these rats with heart failure[67]. Moreover, Niggli's group showed that anti-oxidants (ie, MPG and Mn-cpx3) normalized abnormal RyR sensitivity and hypersensitive E-C coupling in dystrophic cardiomyocytes[68].

Increased oxidative stress might also promote activation of Ca^{2+}/calmodulin-dependent protein kinase, which can phosphorylate RyR2 along with other Ca^{2+} handling proteins, and increase the propensity towards cardiac arrhythmias[69]. We have previously shown that CaMKII phosphorylation of RyR2 leads to increased channel open probability[19]. Recently, we demonstrated that constitutive phosphorylation of RyR2 by CaMKII in RyR2-S2814D knock-in mice promoted abnormal SR Ca^{2+} release events associated with ectopic activity and ventricular arrhythmias[10]. On the other hand, genetic inhibition of RyR2 phosphorylation at S2814 in RyR2-S2814A knock-in mice conferred protection against ventricular arrhythmias in mice with heart failure induced by transverse aortic banding[10]. These studies suggest that inhibition of RyR2 phosphorylation by CaMKII might provide a very specific way of preventing ventricular and also atrial arrhythmias[6]. Moreover, pharmacological inhibition of the enzyme CaMKII itself might also provide protection against arrhythmias[6, 70, 71].

RyR2 is also regulated by protein kinase A (PKA) phosphorylation, and increased PKA phosphorylation of RyR2 has been observed in patients with atrial fibrillation[7]. Shan *et al*[57] demonstrated that mice in which RyR2 was constitutively by PKA (RyR2-S2808D knock-in mice) exhibited an increased open probability, more calcium sparks, and increased SR Ca^{2+} leak. Inhibition of PKA phosphorylation of RyR2 in RyR2-S2808A mice was shown to protect against catecholamine-induced ventricular arrhythmias[72]. Although there are currently no drugs that specifically reduce RyR2 phosphorylation, beta blockers such as carvedilol have been shown to reduce RyR2 phosphorylation and thereby RyR2 open probability in patients with atrial fibrillation[73]. In addition, some beta blockers such as carvedilol also have antioxidant properties in addition to beta-adrenergic blockade, and may be useful in prolongation of arrhythmia-free survival in patients with congestive heart failure versus beta blockers lacking anti-oxidant properties[74]. Clearly, further pharmacological studies would be needed to determine whether modulating post-translational modifications of RyR2 represents a suitable anti-arrhythmic strategy.

Conclusions

Cardiac arrhythmias is a potentially life-threatening complication of genetic and structural heart disease. Recent insights into excitation-contraction coupling have implicated release of SR Ca^{2+} through RyR2 as a key mechanism for the initiation and maintenance of both atrial and ventricular arrhythmias. RyR2-mediated release of SR Ca^{2+} is a tightly regulated process that involves discrete release of Ca^{2+} during systole, and cessation of Ca^{2+} release during diastole. For timely rhythmic release of Ca^{2+} from RyR2, the channel must succinctly open in response to cytoplasmic Ca^{2+} flux, but remain closed during diastolic SR Ca^{2+} filling. Destabilization of RyR2 may occur as a result of genetic mutations (ie, CPVT) or acquired (*eg*, oxidation, nitrosylation, phosphorylation) modifications, resulting in pathologic diastolic Ca^{2+} release, and subsequent arrhythmias.

Given the prominent role of RyR2 in SR Ca^{2+} release, pharmacological strategies to modulate RyR2 stability and gating have shown great promise as a therapy for cardiac arrhythmias. Several drugs targeting RyR2, such as benzothiazepine derivatives and flecainide, bind RyR2 directly and reduce the open probability of RyR2, thereby reducing pathological SR Ca^{2+} "leak". As benzothiazepines and flecainide have an additional role in blockade of voltage-gated sodium channels and delayed rectifier potassium channels, there may be an additive effect on anti-arrhythmic action, however further studies are necessary to evaluate whether this may occur independently of RyR2 blockade or enhancement of RyR2-FKBP12.6 binding. Additionally, dantrolene has been shown to bind directly to RyR2 and stabilize inter-domain regions, although the effects on RyR2 open probability are still controversial. Each of these drugs has a unique mechanism of action, as dantrolene stabilizes N-terminal and central domain interactions, benzothiazepines increase FKBP12.6 (calstabin2) binding to RyR2 (among other mechanisms), and flecainide blocks the open state of the channel. These drugs have proven particularly helpful in CPVT, in which stabilization of RyR2 reduces diastolic SR Ca^{2+} leak, and therefore reduces delayed afterdepolarizations.

In patients with structural heart disease, such as in congestive heart failure, acquired alterations in RyR2 function occur primarily due to post-translational modification of the channel. There is extensive evidence that hyperphosphorylation of RyR2 in heart failure also promotes the occurrence of SR Ca^{2+} leak[10, 18, 72, 75]. By genetic or pharmacological blockade of RyR2 phosphorylation at CaMKII or PKA site, animal models have shown that effective arrhythmia prophylaxis is possible, especially under catecholaminergic conditions or after stress exercise. Recent insights also indicate that adverse redox remodeling of RyR2 may predispose to cardiac arrhythmias. Emerging data suggest that certain beta-adrenergic blocking agents, such as carvedilol, may also exert a redox-stabilizing effect on RyR2, which may potentially increase survival in patients with acquired heart disease. Ultimately, these insights will guide the design of future studies in human patients, whereby stabilization of the RyR2 channel might lead to improved outcomes in morbidity and mortality.

Acknowledgements

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