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Review

Ryanodine receptor studies using genetically engineered mice

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ABSTRACT

Ryanodine receptors (RyR) regulate intracellular Ca^{2+} release in many cell types and have been implicated in a number of inherited human diseases. Over the past 15 years genetically engineered mouse models have been developed to elucidate the role that RyRs play in physiology and pathophysiology. To date these models have implicated RyRs in fundamental biological processes including excitation–contraction coupling and long term plasticity as well as diseases including malignant hyperthermia, cardiac arrhythmias, heart failure, and seizures. In this review we summarize the RyR mouse models and how they have enhanced our understanding of the RyR channels and their roles in cellular physiology and disease.

1. Introduction

RyRs are a family of intracellular Ca^{2+} release channels that mediate the release of Ca^{2+} from the sarco/endoplasmic reticulum (S/ER). RyR1 and RyR2 are found predominantly in skeletal and cardiac muscle, respectively, where they play a central role in excitation–contraction coupling [1–3]. RyR3 is also expressed in skeletal muscle at a low level [4], though its physiologic role there is unclear. All three isoforms are expressed in the mammalian brain. RyR1 is enriched in Purkinje cells of the cerebellum, RyR2 is predominantly expressed in the dentate gyrus of the hippocampus [5], and RyR3 has been detected in the hippocampal CA1 pyramidal cell layer, the basal ganglia, and olfactory bulbs [6]. RyR2 is also found in pancreatic islets [7] and RyR1 and RyR3 have been reported in leukocytes [8,9]. All three isoforms are present in smooth muscle cells [10] (Fig. 1).

RyR1 cDNA was originally cloned in 1989 from rabbit fast twitch skeletal muscle [11,12] and later by a third group in 1990 [13]. In 1990 RyR2 cDNA was sequenced from rabbit heart [14,15] and 2 years after that the cDNA of RyR3 was cloned from a rabbit brain cDNA library [16]. All RyR isoforms exist as homotetramers composed of four ~565 kDa subunits, with the N-terminal 4/5 of the channel comprising an enormous cytoplasmic domain that serves as a scaffold for proteins that regulate the channel function, and the remaining 1/5 in the SR lumen [17]. Studies in which truncated RyR was recombinantly expressed and then reconstituted in planar lipid bilayers have identified the 1030 C-terminal amino acids as capable of forming a channel thereby indicating that the transmembrane regions of the channel are likely included in this region [18]. However, it remains unclear as to how many transmembrane segments exist in the RyR channels with the prevailing view being that there are six for each monomer but possibly as many as eight [19]. The three isoforms share ~65% homology with most of the sequence disparity clustered in three divergent regions referred to as D1, D2, and D3 [17].

Excitation–contraction (EC) coupling in mammalian skeletal muscle is initiated by depolarization of the surface membrane (sarcolemma), which causes conformational changes in plasma membrane Cav1.1 Ca^{2+} channels (L-type Ca^{2+} channels or LTCC). This leads to activation of closely apposed RyR1 channels found on the terminal cisternae of the SR resulting in the rapid release of Ca^{2+} from the SR into the cytosol. Ca^{2+} binds to troponin C enabling the formation of cross bridges between actin and myosin filaments and sarcomere shortening. A direct interaction between amino acid residues 1837–2168 on rabbit RyR1 and a portion of the α_{1S} II–III loop on Cav1.1, is thought to allow for communication between the two channels during EC-coupling [20]. Activation of LTCC also mediates mammalian cardiac EC-coupling, albeit through a different mechanism. T-tubule depolarization leads to Ca^{2+} entry through Cav1.2 and, in a process known as Ca^{2+} induced Ca^{2+} release (CICR), Ca^{2+} binds to RyR2 leading to activation of the channel and release of SR Ca^{2+} stores. After contraction in both skeletal and cardiac muscle, Ca^{2+} is pumped back into the SR through sarco/endoplasmic reticulum ATPase (SERCA) [21].

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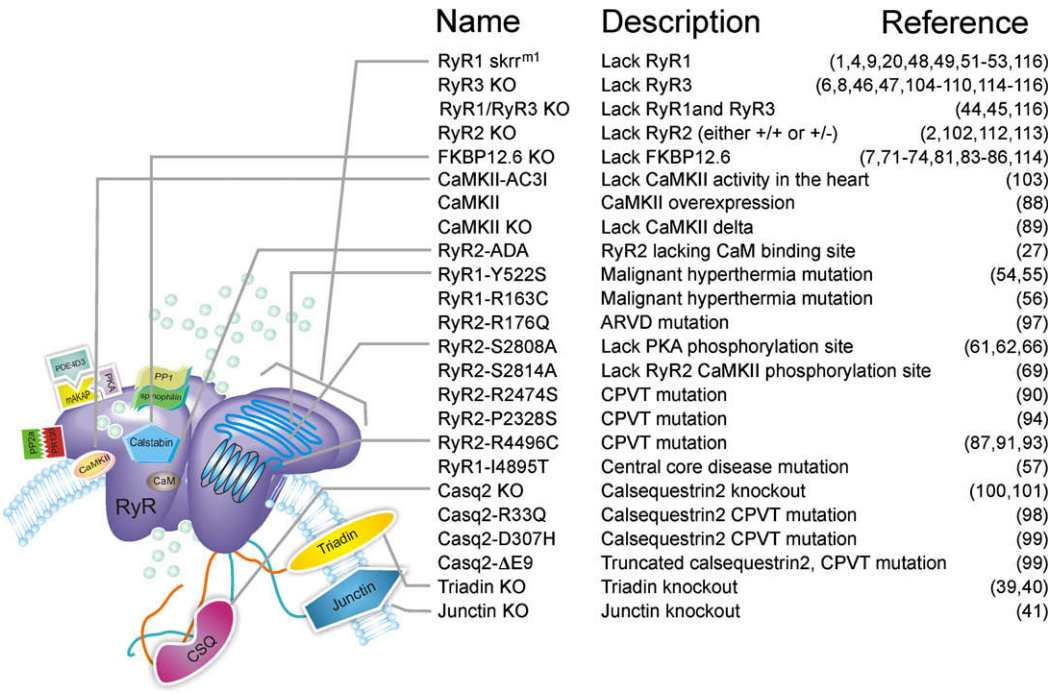

Fig. 1. Genetically modified mouse lines discussed in this review with the RyR targeted mutations highlighted to their respective region of the channel.

The RyR channels are macromolecular complexes that are regulated by enzymes targeted to the channel cytoplasmic domain as well as physiological activators and inhibitors [17]. Ca^{2+}, the most important activator of RyR, activates RyR1–3 at concentrations in the low hundreds of nanomolar [22] and is responsible for RyR2 activation in the heart and amplification of RyR1 Ca^{2+} release in skeletal muscle [3]. At higher concentrations (~1 mM) Ca^{2+} inhibits RyR1 but has less of an inhibitory affect on RyR2 and RyR3 [3]. Whether or not the inhibitory effects of Ca^{2+} on RyRs exist to prevent positive feedback on RyRs (i.e. Ca^{2+} released from RyR would continuously activate the channels and prevent them from closing) remains contentious [3,23].

Magnesium inhibits RyR activity, either by competing with Ca^{2+} or by binding to specific inhibition sites [24,25]. Calmodulin (CaM), a 17 kDa Ca^{2+} binding protein, binds to RyR1 at Cys3635 [26] and to the corresponding region on RyR2, 3578–3603 [27]. CaM can either activate or inhibit RyR1 depending on Ca^{2+} concentrations [28] but appears to preferentially inhibit RyR2 [29]. The RyR cytosolic N-terminal region contains leucine–isoleucine zipper motifs, that function as specific anchoring sites for muscle A-kinase-anchoring protein (mAKAP), which targets PKA and phosphodiesterase 4D3 (PDE4D3) to the channel, as well as for spinophilin and PR130, which target the protein phosphatases PP1 and PP2A to RyR2, respectively [30]. The 12 and 12.6 kDa FK506 binding proteins (FKBP12 and FKBP12.6) bind to and stabilize the closed state of RyR1 [31] and RyR2 [32], respectively, and are essential for coupled gating of RyR channels [33,34]. Now that cellular functions for FKBP12 and FKBP12.6 as components of the RyR1 and RyR2 macromolecular complexes have been identified we have proposed renaming them as calcium channel stabilizing binding proteins, Calstabin1 and Calstabin2, respectively [35]. Ca^{2+}/CaM-dependent protein kinase II (CaMKII) associates with, phosphorylates, and regulates the activity of RyR in the heart [36] and skeletal muscle [37]. Junctin, triadin, and calsequestrin form a complex with the luminal C-terminus of RyR [38]. Calsequestrin, a low affinity, high capacity luminal Ca^{2+} buffering protein regulates local Ca^{2+} levels near the RyR pore and may also directly modulate RyR activity [38]. Triadin, an integral SR membrane protein, is important for proper orientation and activity of the RyR-LTCC Ca^{2+} release unit (CRU) in both cardiac and skeletal muscle, as triadin deficient mice have a 50% reduction in the number of CRUs and those remaining are fragmented, lacking proper SR – T-tubule contact [39,40]. Junctin, an integral SR membrane protein, may play a role in modulating the regulatory action of calsequestrin on RyR1 in skeletal muscle and has been demonstrated to stabilize RyR2 in myocardium using junctin deficient mice [41]. Sorcin has been proposed to interact with RyR to reduce EC-coupling gain and help terminate CICR [42] (Fig. 2).

2. Skeletal muscle

That depolarization of the sarcolemma induces skeletal muscle contraction via SR Ca^{2+} release through RyR1 was originally demonstrated pharmacologically using the RyR blocker ryanodine [43]. The earliest animal data supporting this model came from the RyR1 skrr^{m1} mouse, which was engineered lacking exon 2 of *Ryr1* and expresses non-functional RyR1 [1]. Homozygous skrr^{m1} mice have severe skeletal muscle abnormalities and die perinatally due to respiratory failure, based on the observation that the pups fail to breathe and appear cyanotic after birth.

Isolated neonatal skeletal muscle from the skrr^{m1}/skrr^{m1} mice does not contract in response to electrical stimulation, but does contract in response to caffeine [1], a pharmacological activator of RyRs. The caffeine response is abolished in RyR1/RyR3 double knockout mice suggesting that RyR3 is functionally expressed in neonatal skeletal muscle and is responsible for the residual caffeine response observed in the RyR1 skrr^{m1} skeletal muscle [44,45]. RyR3 expression in skeletal muscle has been shown to decline with age [46] and neonatal but not adult RyR3 knockout mice exhibit impaired skeletal muscle function [47], further supporting the idea that RyR3 plays a more substantial role in neonatal as opposed to adult skeletal muscle. Electron microscopic studies of isolated hind limb and diaphragm muscles show that skrr^{m1}/skrr^{m1} mice are "dyspedic" (lack the cytosplasmic domains of RyR called

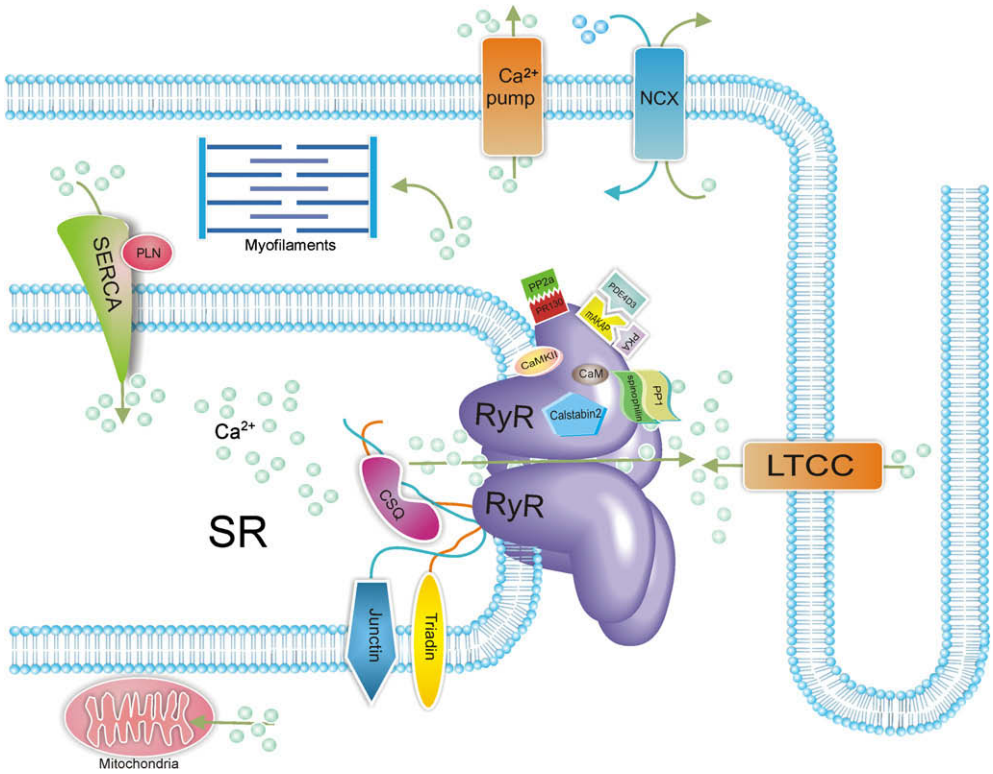

Fig. 2. Cardiac excitation–contraction coupling. Ca^{2+} entry through LTCC activates RyR2 to release sarcoplasmic reticulum (SR) Ca^{2+} stores into the cytosol where the Ca^{2+} binds to myofilaments leading to cell shortening and cardiac contraction. Ca^{2+} is subsequently pumped back into the SR through SERCA and out of the cell through the sodium–calcium exchanger and the plasmalemmal Ca^{2+} pump. RyR2 is a homotetramer regulated by many proteins (for simplicity these proteins are depicted here on only one RyR monomer).

"feet") and also lack tetrads (RyR1–Cav1.1 complexes) [48], providing additional evidence that these structures play key roles in skeletal muscle EC-coupling.

The ability to reconstitute recombinant RyR in the dyspedic myotubes isolated from the RyR1 deficient mice has helped elucidate several RyR related processes including the crosstalk between RyR1 and Cav1.1 [49], diseases associated with RyR1 mutations [50], as well as the effect of modulatory proteins such as CaM on RyR1 [51]. For example, myotubes isolated from RyR1 deficient mice have a 40% reduction in the surface expression of Cav1.1 with the remaining channels exhibiting reduced Ca^{2+} conductance as well as altered kinetics in response to the Cav1.1 activator Bay K 8644 [52]. Transfection of these myotubes with recombinant RyR1 restores Cav1.1 function back to normal [49] indicating that RyR1 acts as an allosteric modulator of Cav1.1 [53].

Transgenic RyR1 mice have helped elucidate the pathophysiology of malignant hyperthermia (MH), a pharmacogenetic condition whereby a mutation carrying patient who receives a volatile anesthetic suddenly deteriorates into a hypermetabolic state with skeletal muscle contracture and hyperthermia. More than 90 mutations in RyR1 are linked to MH susceptibility. MH is believed to be caused by anesthesia induced dysregulation of myoplasmic Ca^{2+} handling and that the efficacy of dantrolene, the only drug available for treating MH, is based on its ability to inhibit RyR1 and stabilize Ca^{2+} levels [50].

Mice homozygous for the RyR1-Y522S mutation, a mouse model of MH, die between embryonic day 17.5 and postnatal day 1 and have marked skeletal abnormalities [54]. Heterozygous mice are born without any overt defects and develop MH when exposed to isoflurane (a volatile anesthetic), as characterized by full body contracture and elevated body temperature. Skeletal muscle isolated from heterozygous mice has increased sensitivity to caffeine suggesting that the lethal phenotype imparted by this mutation may be related to higher sensitivity of RyR1 to cytosolic Ca^{2+} resulting in RyR1 openings during rest, referred to as SR Ca^{2+} leak.

The RyR1-Y522S mice have helped elucidate a putative pathophysiological mechanism for heat stroke [55]. Skeletal muscle myocytes isolated from the RyR1-Y522S mice have increased SR Ca^{2+} leak, which leads to the generation of reactive nitrogen species and S-nitrosylation of RyR1, which further enhances SR Ca^{2+} leak through RyR1. This feed forward process results in increased temperature sensitivity of RyR1 for activation causing muscle contractures upon exposure to elevated temperatures and eventual mitochondrial damage and myopathy.

The RyR1-R163C mice are another model of MH [56]. Homozygous R163C mice are embryonically lethal while heterozygous mice are viable and develop MH when exposed to low doses of vaporized halothane. None of the R163C mice develop signs of MH when pretreated with dantrolene. Myotubes isolated from both homozygous and heterozygous R163C knock-in mice have increased sensitivity to caffeine similar to that observed in the Y522S mice. However, in contrast to the normal baseline Ca^{2+} levels in heterozygous Y522S mice, the resting Ca^{2+} levels in heterozygous R163C mice are elevated compared to control mice suggesting that the R163C mutation located nearer to the N-terminus of RyR1 has a greater affect on Ca^{2+} gating and leak.

Mutations in RyR1 have also been linked to central core disease (CCD), a congenital myopathy characterized by poor muscle tone, muscle weakness, and musculoskeletal abnormalities throughout infancy and adulthood [50]. Mice engineered with one of these mutations, RyR1-I4895T, die shortly after birth due to cyanosis secondary to severe skeletal muscle abnormalities [57]. Histologically, skeletal muscle isolated from these mice is not robustly striated and exhibit poorly aligned myotubes. Myotubes isolated from

these mice also lack voltage induced Ca^{2+} release despite having a normal SR load and no SR Ca^{2+} leak, suggesting that the pathophysiology of this CCD mutation is linked to defects in Cav1.1 induced activation of RyR1 (un-coupling) rather than leaky RyR1 [57]. These mice have normal retrograde coupling of RyR1 and Cav1.1 suggesting that orthograde and retrograde communication between RyR1 and Cav1.1 operate through independent pathways.

3. Heart

The primary role that RyRs play in cardiac CICR and EC-coupling was originally demonstrated using ryanodine [58], which was found to reduce contractility by blocking SR Ca^{2+} release [59]. The development of an RyR2 knockout mouse, which is embryonically lethal and whose cardiomyocytes lack SR Ca^{2+} release, provides additional evidence that this isoform is the major source of SR Ca^{2+} release during cardiac EC-coupling [2].

Despite the central role that Ca^{2+} release via RyR2 plays in mammalian cardiac EC-coupling the role that physiologic modulation of RyR2 has in homeostasis is less clear. One hypothesis, that PKA phosphorylation of RyR2 is a downstream mediator of the "fight or flight" response has generated significant controversy. During the normal sympathetic response norepinephrine is released from sympathetic nerve terminals and binds to and stimulates the β_1-adrenergic receptor, a G-protein coupled receptor, leading to activation of PKA. PKA subsequently phosphorylates many Ca^{2+} handling proteins including LTCC, phospholamban (PLN), and RyR2, generally resulting in faster Ca^{2+} transients with higher amplitude [21]. PKA phosphorylation of PLN at Ser16 relieves its inhibitory effect on SERCA and consequently accelerates SR Ca^{2+} loading [60], while PKA phosphorylation of RyR2 at Ser2808 increases RyR2 open probability, which can potentially increase SR Ca^{2+} release [17]. However, while transgenic mice with PLN that cannot be PKA phosphorylated have a blunted cardiac response to β_1-adrenergic stimulation [60], it has been reported that mice with an RyR2-S2808A mutation have largely normal responses [61,62], however these results have been disputed. Indeed, evidence exists that modulation of RyR2 plays a role in the response to β_1-adrenergic stimulation [63,64], warranting further investigations as to whether these RyR2 phosphorylation modulates peak Ca^{2+} transient amplitude and cardiac contractility [65]. The existence and functional significance of an alternate RyR2 PKA site at Ser2030 is also controversial [62,66–68].

CaMKII dependent phosphorylation of RyR2 at Ser2814 has also been shown to increase the Ca^{2+} sensitivity and open probability of RyR2 [17] leading to the idea that the "fight or flight" response is mediated by β_1-adrenergic activation of CaMKII and subsequent phosphorylation of RyR2. The recent development of the RyR2-S2814A transgenic mouse harboring RyR2 lacking the major CaMKII phosphorylation site, will help elucidate this hypothesis [69]. The RyR2-S2814A mouse will also make it possible to test whether CaMKII dependent phosphorylation of RyR2 is involved in the force frequency response (FFR). Since the late 19th century it has been observed that cardiac contractility increases as a function of contractile frequency. The ability of CaMKII to sense the frequency of Ca^{2+} oscillations has led to the hypothesis that frequency dependent activation of CaMKII and phosphorylation of RyR2 at Ser2814 contributes to the FFR [70].

Heart failure is a syndrome characterized by the inability of the heart to produce sufficient cardiac output to meet the metabolic needs of the organs. A hallmark of heart failure is elevation of circulating catecholamines, which leads to persistent stimulation of the β_1-adrenergic receptor. Our laboratory has reported that PKA hyperphosphorylation of RyR2 at Ser2808, and depletion of calstabin2 from the channel results in defective RyR2 channels that leak SR Ca^{2+} contributing to heart failure progression [32] and arrhythmias [71]. The term "PKA hyperphosphorylation" refers to RyR2 with three to four of the four monomers that comprise this homotetrameric channel phosphorylated by PKA. Depletion of calstabin2 causes increased diastolic leak through the channel [71], leading to depletion of SR Ca^{2+} stores and reduced contractility [72]. Diastolic leak increases I_{TI} (transient inward current) through the Na^+/Ca^{2+} exchanger (NCX), which leads to delayed-after-depolarizations (DADs) [71,73] that can trigger ventricular arrhythmias. In support of these findings, RyR2-S2808A knock-in mice, which cannot be PKA phosphorylated on RyR2, exhibited significantly improved cardiac contractility compared with control mice at 28 days post-myocardial infarction (MI) [66].

Other laboratories, however, have not been able to demonstrate that depletion of calstabin2 from RyR2 induces changes in channel function [74] or that PKA phosphorylation causes dissociation of calstabin1 or 2 from RyR1 or 2, respectively [75,76]. In several instances the explanation for the differences in the results may be due to altered stoichiometry of calstabin and RyR (i.e. addition of molar excesses of calstabin to the immunoprecipitation reactions) [76]. Of particular interest is the observation by Hamilton and colleagues [77], later confirmed by our own studies [78], that nitrosylation of RyR1 can cause dissociation of calstabin1 from the channel. Additionally, oxidation of RyR2 has been shown to disrupt calstabin2 binding to the channel [79] and contribute to heart failure progression [80]. This raises the possibility that the status of nitrosylation and oxidation of the channel may vary from experiment to experiment. As this is a factor that is not typically assessed, differences in nitrosylation or oxidation of the RyR channels could add to the variability of these types of experiments and explain some of the inability of other laboratories to reproduce our findings. In other instances it appears that the inability to reproduce our results may in actuality be explained simply by differences interpretation of the data. For example, in another study, despite concluding that the RyR2-S2808A mutation does not confer cardioprotection against cardiomyopathy, the investigators reported that control mice with cardiomyopathies, induced by aortic banding, exhibited a 20% reduction in cardiac fractional shortening (FS), whereas the RyR2-S2808A mice had no decrease in FS [62,65], and therefore were protected against progression of cardiac dysfunction in agreement with our findings [66].

Animal data supporting the hypothesis that depletion of calstabin2 from RyR2 increases susceptibility to arrhythmias comes from the calstabin2$^{-/-}$ and calstabin2$^{+/-}$ mice, which have increased rates of exercise-induced sudden cardiac death (SCD) [71] and atrial fibrillation [81]. This increased incidence of SCD can be eliminated in calstabin2$^{+/-}$ mice treated with JTV519, a 1,4-benzothiazepine that facilitates the re-association of calstabin2 to RyR2. Although some investigators using cellular based studies have suggested that the cardioprotective effects of JTV519 are mediated through pathways independent of calstabin2 [82] in our in vivo studies we found that JTV519 was not effective in preventing SCD in calstabin2$^{-/-}$ mice indicating that the drug requires calstabin2 for its mechanism of action in the heart [83]. Additionally, control mice treated with JTV519 had improved contractility post-MI while calstabin2$^{-/-}$ mice did not [72]. On the cellular level, cardiomyocytes isolated from calstabin2$^{-/-}$ mice have increased Ca^{2+} spark amplitude and size [84] and greater SR Ca^{2+} leak [81]. Other laboratories have similarly reported that calstabin2 deficiency is linked to enhanced susceptibility to arrhythmias [81,85,86]. However, once again others have not been able to reproduce these results [74,87]. As is often the case with in vivo studies, differences in the conditions used to elicit the arrhythmias are the most likely explanation for the ability of some groups but not others to observe stress induced arrhythmias in the calstabin2 deficient mice. In addition, differences in genetic background often cause phenotypic differences in mice. In our studies mice are back-

crossed more than six generations into the C57BL/6J genotype to ensure that all genetically altered mice are evaluated in the same genetic background. In the study that reported no increased susceptibility to arrhythmias in a calstabin2 deficient mouse model there was no mention of the genetic background of the mice [74]. These conflicting reports highlight the fact that animal models, while helpful, do not necessarily always solve controversies.

Constitutive CaMKII phosphorylation of RyR2 has also been hypothesized to play a role in the pathogenesis of heart failure. This is based on observations that transgenic mice overexpressing CaMKII have increased CaMKII dependent phosphorylation of RyR2 and develop cardiac hypertrophy as well as signs of dilated cardiomyopathy [88]. Additionally, CaMKII delta deficient mice are protected against pressure overload induced cardiomyopathy [89]. It is believed that the hyperadrenergic state induced by these conditions leads to chronic activation of β-adrenergic receptors leading to downstream activation of CaMKII, which then phosphorylates RyR2, causing a diastolic leak through the channel, resulting in depleted SR Ca^{2+} stores and reduced cardiac function.

A role for CaMKII phosphorylation of RyR2 in arrhythmias comes from a study showing that atria from patients with AF have elevated levels of activated CaMKII and increased phosphorylation of RyR2 at Ser2815 (corresponding to Ser2814 in mice) [69]. Furthermore, mice engineered with a RyR2-S2814A mutation have been reported to be protected from developing AF when exposed to high frequency pacing, which activates CaMKII, in the presence of the cholinomimetic carbachol [69].

CaM regulation of RyR2 may play an important role in preventing cardiomyopathy and heart failure. The RyR2-W3587A/L3591D/F3603A (RyR2-ADA) knock-in mice, which have an altered CaM binding domain on RyR2, develop left ventricular hypertrophy shortly after birth and begin to die around post natal day 10 [27]. The investigators in this study attributed the hypertrophy and death to enhanced phosphorylation of class II histone deacetylases (HDACs) by an unidentified Ca^{2+} dependent kinase, which leads to increased activity of growth related transcription factors. The inability of CaM to assist in terminating systolic RyR2 Ca^{2+} release in the mutant mice leads to prolonged Ca^{2+} transients and elevated cytosolic [Ca^{2+}], which activates the kinases responsible for phosphorylating the HDACs [27].

Transgenic RyR2 mice have helped elucidate the pathophysiology of a pair of rare familiar arrhythmias: catecholaminergic polymorphic ventricular tachycardia (CPVT) and arrhythmogenic right ventricular dysplasia type 2 (ARVD2). Patients carrying CPVT mutations have no signs or symptoms at baseline but are at increased risk for exercise induced sudden cardiac death (often the presenting symptom). In ARVD, these arrhythmias are associated with fibrofatty replacement of the right ventricle [17]. To date more then 70 mutations in three regions of RyR2 have been found in patients with CPVT and ARVD. RyR2 channels engineered with some of these mutations have been studied in planar lipid bilayers and exhibited normal open probabilities at diastolic [Ca^{2+}] (150 nM), but increased open probabilities under similar conditions after PKA phosphorylation [71]. These results have led to the hypothesis that activation of β_1-adrenergic receptors during exercise leads to PKA dependent phosphorylation of RyR2 that unmasks a gain-of-function mutation in the channel, which combined with the increased SR Ca^{2+} load due to higher activated PLN, leads to diastolic leak, I_{TI}, and DADs [71].

Support for this hypothesis comes from the RyR2-R2474S mouse, which reproduces many aspects of human CPVT [90]. First, homozygous mutants do not follow Mendelian inheritance patterns after embryonic day 16.5 suggesting that this mutation can cause intrauterine lethality. Moreover, the embryonic lethality was prevented by treating the mothers with S107, a novel drug similar to JTV519 that prevents diastolic SR Ca^{2+} leak via RyR2 by enhancing the binding affinity of calstabin2 for the mutant channels [90]. At rest the RyR2-R2474S mice have normal heart rates and rhythm, however when exercised and treated with epinephrine these mice developed bidirectional and polymorphic ventricular tachycardia, similar to patients with CPVT. Ventricular cardiomyocytes isolated from these mice had increased I_{TI} densities and Ca^{2+} sparks when treated with isoproterenol but not at rest. Treating these mice with S107 reduced I_{TI} density and the incidence of ventricular tachycardias [90].

The RyR2-R4496C knock-in mouse is another animal model of CPVT. These mice are different from the RyR2-R2474S mouse in that they demonstrated a significant increase in spontaneous opening of RyR2 even in the absence of isoproterenol [91]. Additionally, SR microsomes isolated from these mice treated with epinephrine showed no reduction in calstabin2 binding suggesting that β1-adrenergic receptor activation does not cause dissociation of calstabin2 from RyR2 [87]. Spark frequency in cardiomyocytes isolated from these mice increased rapidly in response to increasing Ca^{2+} concentrations compared to control mice [91] in agreement with the concept that the underlying cause of arrhythmias in this CPVT mutant is increased sensitivity of RyR2 to Ca^{2+} [71]. In this study the investigators suggest that the CPVT mutations increase the sensitivity of RyR2 to luminal [Ca^{2+}], as opposed to cytosolic [Ca^{2+}], and that during exercise, stimulation of the β_1-adrenergic receptor pathway causes PKA dependent phosphorylation of PLN, increased SERCA activity, and increased SR Ca^{2+} load, which leads to enhanced opening of RyR2 and increased SR Ca^{2+} efflux during diastole, causing DADs [91]. This hypothesis, store overload induced calcium release (SOICR), was originally developed using a heterologous system whereby investigators observed the number of cells exhibiting Ca^{2+} oscillations in response to progressively elevated SR Ca^{2+} stores [92]. In these studies the investigators found that HEK cells expressing recombinant RyR2 channels containing CPVT mutations were more sensitive to elevated SR Ca^{2+} load then WT controls [92]. The observation that the RyR2-R4496C mice have increased frequency of Ca^{2+} waves and sparks at lower SR Ca^{2+} loads may support the SOICR hypothesis [91].

Optical mapping studies using hearts from RyR2-R4496C mice have been useful in elucidating the mechanism of arrhythmogenesis in CPVT [93]. The arrhythmias appear to originate within the His-Purkinje conduction system and subsequently become reentrant tachycardias and ventricular fibrillation. Patch clamp experiments demonstrated that isolated Purkinje cells from the mutant mice have a higher frequency of DADs that is exacerbated by application of isoproterenol [93]. It remains to be seen whether other CPVT mouse models have similar conduction abnormalities and whether PKA or CaMKII phosphorylation of RyR2 plays a role.

CPVT mutations also exist in the central region of RyR2. To test the effect of these mutations a mouse line with the RyR2-P2328S CPVT mutation has been generated. These animals have sustained runs of ventricular tachycardia when subjected to programmed electrical stimulation protocols in the presence of isoproterenol. Additionally, in the presence of isoproterenol, isolated cardiomyocytes from the homozygous P2328S mice exhibited altered Ca^{2+} homeostasis, characterized by ectopic Ca^{2+} release peaks during 0.5 hz stimulation [94].

Dysruption of interdomain interactions within the RyR2 complex has also been proposed as a mechanism underlying CPVT [95]. In this model RyR2 channels with CPVT mutations have normal domain–domian interactions at baseline, however β-adrenergic stimulation leads to unzipping of these domains, which causes the SR Ca^{2+} leak responsible for DADs [96]. Accordingly, it has been suggested that JTV519 prevents SR Ca^{2+} leak by stabilizing the zipped state of the channel [96].

The RyR2-R176Q mouse, the only published mouse model of ARVD2, has been more difficult to reconcile with the human disease. RyR2-R176Q mice are similar to ARVD2 patients in that they demonstrate right ventricular diastolic dysfunction and β-adrenergic receptor activation induced premature ventricular beats, however they have no overt histological changes [97]. One possible explanation is that ARVD patients carrying the RyR2-R176Q mutation also carry a RyR2-T2505M mutation and therefore it is possible that both mutations are required in order for overt histological changes to develop.

Mutations in calsequestrin2 have also been linked to CPVT. Mice engineered to express human CPVT mutations CASQ2-R33Q [98], CASQ2-D307H, and CASQ2$^{\Delta E9}$ (truncated calsequestrin) [99] all exhibit stress induced ventricular arrhythmias as well as reduced levels of calsequestrin, due to decreased stability of the mutant proteins. Cardiomyocytes isolated from these mice have enhanced RyR2 mediated SR Ca^{2+} leak, as well as DADs [98,99], and EADs [98]. Calsequestrin knockout mice have normal baseline heart function and develop sustained runs of ventricular tachycardia during exercise [100,101]. Cardiomyocytes isolated from these mice have a left shifted SR leak/load relationship whereby a relatively low SR Ca^{2+} load induces a large SR Ca^{2+} leak through RyR2. These data suggest that arrhythmogenesis in CPVT patients carrying calsequestrin mutations may be due to diminished calsequestrin dependent modulation of RyR2 secondary to reduced total calsequestrin protein levels and perhaps lower affinity of calsequestrin to RyR2.

A putative role for RyR2 mediated Ca^{2+} release in automaticity has been demonstrated in embryonic stem cell-derived cardiomyocytes isolated from RyR2 deficient mice, which lack spontaneous contractions [102]. These observations contribute to the hypothesis that in sinoatrial node (SAN) cells SR Ca^{2+} release through RyR2 activates the NCX producing an inward current that accelerates the rate of spontaneous membrane depolarization that drives the heart rate. As a corollary, phosphorylation of RyR2 by either CaMKII [103] or PKA may mediate the chronotropic response observed during the "fight or flight" response.

4. Brain

Despite the presence of RyRs throughout the brain little is known regarding their functional significance in this organ. The viability of the general RyR3 knockout mouse, in contrast to the RyR1 and RyR2 knockout mice [1,2,104], has resulted in many studies characterizing the neurological phenotype of these mice. The RyR3 deficient mice exhibit decreased social behavior [105] and two times greater locomotor activity [104] that is associated with the tendency to run in circular motions [6]. Striatum from these mice release less dopamine when challenged with low concentration ryanodine, which activates ryanodine receptors, suggesting the possibility that the effect of RyR3 on locomotion may be related to its role in the basal ganglia [106].

Field recordings from the CA1 region of the hippocampus show that RyR3 deficient mice have impaired maintenance of long term potentiation (LTP) [107] when induced by weak stimuli but are similar to controls when LTP is induced via stronger stimulation protocols [6]. These data suggest that RyR3 enhances LTP. Further evidence that RyR3 enhances LTP comes from observations that RyR3 deficient mice have smaller AMPA receptor induced excitatory post-synaptic potentials (these events are an important component of LTP) [107]. Other studies have shown that RyR3 deficient mice have a lower threshold for inducing LTP [108], suggesting that RyR3 naturally inhibits LTP. Furthermore, LTP in RyR3 knockout mice is NMDA independent and the absence of any compensatory mechanisms has led to suggestions that the physiologic role of NMDA receptor activation is to overcome the inhibitory effects of RyR3 on LTP [108].

In Morris water-maze RyR3 knockout mice form superior spatial maps and spend more time in the quadrant containing the hidden platform [108], however once a spatial map has been formed these mice have difficulty learning the position of a relocated platform [6]. The redundancy of RyR expression, specifically in the hippocampus has thus far made it difficult to study the effects of deletion of any one isoform on neuronal plasticity and LTP. The generation of brain specific RyR1 and RyR2 knockouts crossed with RyR3 deficient mice will help overcome this problem.

RyR3 may also play a role in fear conditioning through its ability to activate CaMKII. During the retrieval stages of contextual fear conditioning hippocampal activity of CaMKIIα and CaMKIIβ increases in control mice but not in RyR3 deficient mice. Functionally RyR3 knockout mice have higher locomotor activity after contextual fear conditioning and also spend more time in the open arms of the elevated plus maze [109] suggesting defects in fear conditioning.

Neurons are not the only cells in the brain that express RyRs. Astrocytes, which play key roles in extracellular ion balance, wound healing, inflammation, and glial scar formation, express RyR3 but not RyR1 or RyR2 [110]. Astrocyte motility, measured using the in-vitro wound healing assay, is impaired in both astrocytes isolated from wild type mice treated with high concentration of ryanodine that blocks ryanodine receptors as well as in astrocytes isolated from RyR3 deficient mice [110].

A role for leaky ryanodine receptors in the pathogenesis of epilepsy has recently been described in the RyR2-R2474S mouse model of CPVT [90]. Case records from patients with CPVT arrhythmias include descriptions of syncopal events associated with convulsive movements. These events were attributed to the arrhythmias causing rapid compromise of cerebral blood supply, a phenomenon known as Stokes–Adams attacks. Simultaneous ECG–EEG recordings in RyR2-R2474S mice, however, showed seizure activity without any preceding or concurrent arrhythmias suggesting that the neurological manifestations of the RyR2-R2474S mutation are distinct from the cardiac phenotype. The brains from these mice are histologically normal and cells in the principal hippocampal CA3 layer exhibited profound burst activity suggesting that seizure activity may originate in this region [90]. One question raised by this study is that RyR2 channels with the R2474S exhibited normal single channel properties unless they were PKA phosphorylated to expose the gain-of-function phenotype of this mutation [71]. While strenuous exercise combined with intra-peritoneal injection of epinephrine is required to induce cardiac arrhythmias, the seizures in RyR2-S2474S mice develop spontaneously without any apparent β_1-adrenergic receptor stimulation. Further investigation is needed to better understand how RyR2-R2474S mutations can promote seizures. Additionally, the relationship between RyR Ca^{2+} leak, burst activity, and seizure genesis in neurons remains to be determined.

5. Smooth muscle

Localized RyR mediated SR Ca^{2+} release events called Ca^{2+} sparks activate plasmalemmal K^+ (BK) channels in smooth muscle, inducing spontaneous transient outward currents that hyperpolarize the membrane causing muscle relaxation [111]. Smooth muscle cells isolated from the RyR2 heterozygous knockout mice have reduced Ca^{2+} spark activity [112,113] while cells isolated from the calstabin2 deficient mice, which have leaky RyR2 [81], have increased spark activity [114] suggesting that RyR2 plays an important role in Ca^{2+} spark activity in smooth muscle.

The role of RyR3 in Ca^{2+} spark activity is controversial. One study found that RyR3 deficient mice have reduced smooth muscle tone associated with an increase in Ca^{2+} spark activity, suggesting that RyR3 naturally inhibits Ca^{2+} sparks [115]. However, other

studies did not find any differences in smooth muscle contractile properties or Ca^{2+} spark activity in the RyR3 deficient mice [104,114]. The differences between these studies may be due to the types of smooth muscle used (arterial, bladder).

6. Non-excitable cells

Ca^{2+} handling plays a key role in immune cell functions such as maturation and migration during immune responses. Human Jurkat T-lymphocytes have been reported to express RyR3 [8], however, despite cellular data suggesting a role of RyR3 in lymphocyte proliferation RyR3 deficient mice have normal populations of T- and B-lymphocytes in spleen, thymus, and bone marrow [104]. Similarly, while RyR1 is reportedly expressed in dendritic cells, dendritic cells isolated from RyR1 deficient mice exhibit normal responses to inflammatory stimuli and to stimulate a T cell response [9].

In the liver, electron microscopy studies reveal that hepatocytes isolated from RyR1/RyR3 double knockout mice have large accumulations of glycogen granules leading to hepatomegaly [116]. One proposed explanation for this is that these mice have enlarged adrenal medullas (the adrenal gland modulates glucagon levels via glucocorticoid secretion) as well as increased numbers of lysosomes in medullary cortical cells. However, linking the hepatic changes to the altered adrenal gland morphology is difficult since the RyR1, RyR3 and RyR1/RyR3 deficient mice all have similar adrenal morphologies, yet only the RyR1/RyR3 deficient mice demonstrate any liver abnormalities [116].

RyR2 has also been implicated in insulin secretion from pancreatic beta cells. Calstabin2 deficient mice have elevated blood glucose levels after glucose injection secondary to impaired insulin secretion [8]. Beta-cells from these mice are morphologically similar to control mice and have normal insulin stores. It is hypothesized that cADPR, which is elevated when glucose metabolism is increased, competes calstabin2 off of RyR2 resulting in enhanced open probability of the channel, causing elevations in cytosolic $[Ca^{2+}]$ that trigger insulin secretion. According to this hypothesis it is unclear why insulin secretion is reduced rather than elevated in these mice since the complete absence of calstabin2 should result in greater SR Ca^{2+} leak and higher cytosolic $[Ca^{2+}]$, enhancing the trigger for insulin secretion. More studies aimed at examining the dynamics of intracellular Ca^{2+} handling in these cells are needed to address this issue.

7. Outlook

The development and analysis of genetically engineered RyR mouse models has led to an explosion in our understanding of the physiological and pathological relevance of this complex ion channel. Despite the many answers that these animals have provided there are many more questions that they can be used to address including: (1) what is the physiological role of RyR channels in smooth muscle, are they involved only in relaxation, or also in contraction; (2) what is the biological role for RyR1 and RyR2 in the brain; (3) are RyR channels in pancreatic beta cells a potential therapeutic target for diabetes; (4) what is the role of RyR channels in the immune system? Genetically altered mouse models hold the promise for revealing new understandings of these and many other questions that can only be addressed using in vivo models.

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