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Commentary

Ryanodine receptor calcium channels and their partners as drug targets☆

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ABSTRACT

Ryanodine receptors (RyRs) are high conductance intracellular cation channels that release calcium ions from stores such as the endoplasmic reticulum and sarcoplasmic reticulum. Although RyRs are expressed in many cell types, their roles have only been extensively characterised in tissues in which they are abundant: RyR1 is essential for excitation–contraction coupling in skeletal muscle; whereas RyR2 is required for the analogous signal transduction pathway in heart. Defects in RyR1 cause malignant hyperthermia and a spectrum of myopathies in skeletal muscle; whereas RyR2 dysregulation can result in fatal cardiac arrhythmias and is involved in heart failure. Altered RyR gating has been implicated in a range of other diseases, including epilepsy, neurodegeneration, pain and cancer. RyRs interact with a range of toxic substances, providing insights into their functional and structural properties. Consequently, these channel complexes represent potential therapeutic targets for treatment of numerous diseases. Furthermore, strategies for combating multicellular parasites and agricultural pests could exploit pharmacological differences between their RyRs and those of vertebrates. However, available pharmacological tools for manipulation of RyR gating are generally unsuitable for clinical, veterinary or agricultural use, owing to their lack of selectivity, inappropriate solubility in the aqueous or lipid environment, or generation of side-effects. The expression, subcellular distribution and gating of RyRs is modified by a wide variety of cellular proteins, some of which are expressed in a developmentally or tissue-restricted manner. This commentary examines the possibility of manipulating the expression and function of such proteins in order develop new drugs acting on RyR channel complexes.

1. Introduction: what are ryanodine receptors?

The ryanodine receptors (RyRs) are a family of high conductance cation channels that release Ca^{2+} from intracellular stores such as the endoplasmic reticulum (ER) and sarcoplasmic reticulum (SR). Historically, research on these channels has centred on their roles in excitation–contraction (EC-) coupling in striated muscles, a process in which they play fundamental roles. Mammalian RyRs are encoded by three distinct genes, the products of which share ~65% identity with one another and are posttranscriptionally and posttranslationally modified, generating structural and functional diversity [1]. RyRs are gated by a wide range of endogenous ligands, including Ca^{2+} at nanomolar to micromolar concentrations (Ca^{2+}-induced Ca^{2+}-release), allosteric interactions with L-type voltage-dependent Ca^{2+}-channels, ATP and possibly cyclic ADP ribose (cADPr), a second messenger generated by ADP ribosyl cyclases [2]. Endogenous inhibitors of these channels include Ca^{2+} at high micromolar concentrations and millimolar Mg^{2+}.

Type 1 RyR (RyR1) is expressed at greatest levels in the SR terminal cisternae of skeletal muscle. Ryanodine is a plant alkaloid displaying high affinity interactions with RyRs, which modify their intrinsic channels in a complex, 'use-dependent' manner: activating them at submicromolar concentrations, locking them into a subconductance state at higher levels and closing them at millimolar concentrations [3]. Generation of tritiated ryanodine facilitated biochemical isolation and characterisation of RyR1 protein from skeletal muscle [4,5]. RyR1 is a tetramer of 565 kDa subunits in combination with a range of accessory proteins, making it one of the largest channel complexes identified. The essential function of these channels was demonstrated in transgenic mice lacking functional copies of the *RYR1* gene: these animals die perinatally due to asphyxia, owing to defective EC-coupling in the diaphragm [6].

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Abbreviations: FKBP12, FKBP12.6, 12/12.6 kDa FK506-binding proteins; AKAP, a-kinase anchoring protein; AVRD2, arrhythmogenic right ventricular dysplasia type 2; CASQ, calsequestrin; CREB, cAMP response element binding protein; CREM, cAMP response element modulator; CPVT1, catecholaminergic polymorphic ventricular tachycardia type 1; CCD, central core disease; ER, endoplasmic reticulum; EC-, excitation–contraction; HF, heart failure; $InsP_3Rs$, inositol 1,4,5-trisphosphate receptors; MHS, malignant hyperthermia susceptibility; MTMR14, myotubularin-related protein 14; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; Sp1, specificity protein-1.

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RyR2 is expressed at greatest abundance in the SR of mammalian heart, from which it was first biochemically isolated [7]. Numerous biochemical, physiological, molecular and pharmacological studies support a critical role of RyR2 in cardiomyocyte EC-coupling [8]. Transgenic mice lacking functional *RYR2* expression die *in utero*, displaying defective heart tube formation [9], implying a role for these channels in developmental processes. RyR2 is also expressed at relatively high levels in the nervous system and at lower abundance in several other tissues and cell types [10,11]. The roles of RyR2 in non-muscle tissues have not been extensively characterised. However, antisense oligonucleotide reduction of expression of the mRNA encoding RyR2 in mice indicates that this ion channel participates in memory processing [12] and depressive states [13], but not in muscarinic antinociception [14]. Such down-regulation of RyR2 protein expression in the central nervous system caused selective, reversible effects on memory and depressive states, indicating that this channel represents a promising candidate for the development of drugs for treatment of mood or memory disorders.

RyR3 displays a low-level, widespread tissue distribution, being most abundant in brain and in certain skeletal muscles [10]. Transgenic mice lacking *RYR3* are viable and fertile, but display hyperactivity and deficits in contextual fear conditioning [15]. A recent in-depth behavioural examination of these transgenic mice revealed that they also display decreased social interaction relative to their wild type littermates [16]. Antisense oligonucleotide studies in mice indicate that RyR3 plays roles in muscarinic antinociception [12], depression [13] and memory processing [14]. Upregulation of RyR3 is reported to be neuroprotective in a mouse model of Alzheimer's disease [17]. Together these findings indicate that RyR3 is a promising candidate for the development of therapeutic strategies for treatment of mood disorders, memory dysfunction, neurodegeneration or acute pain, particularly given the apparent non-essential role of this channel in normal physiology revealed in transgenic knockout or antisense knockdown mice.

2. Diseases, drugs and commercial exploitation: which targets?

Ca^{2+} is a key second messenger, regulating numerous physiological and pathological processes [18]. RyRs show considerably larger conductances (~100 pS for pCa^{2+}) and longer open durations per opening event than most other cation channels, including their relatives and neighbours in the ER, the inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$). Consequently RyRs release large quantities of Ca^{2+} per opening event (about 20-fold more per individual channel complex than $InsP_3Rs$) [3], indicating that despite being present at lower abundance than $InsP_3Rs$ in most cell types, they are suited to play specialised roles in Ca^{2+} signalling pathways, as is the case in striated muscle EC-coupling. Such specialisation is further enhanced by the restricted subcellular localisation of these channels in certain tissues. RyRs are also a convergence point for many intracellular signals, which is extended via interactions with a plethora of accessory proteins [19]. As a result, defects within RyR complexes leading to dysregulated channel gating cause disease.

Several inherited RyR associated diseases have been extensively characterised to date. Malignant hyperthermia (MH) susceptibility is a pharmacogenetic disorder of skeletal muscle in humans and other vertebrates, in which certain halogenated or depolarising anaesthetics trigger dysregulated release of Ca^{2+} from the SR, causing sustained activation of the contractile apparatus, muscular rigidity and hyperthermia. Untreated MH episodes can result in rhabdomyolysis, organ failure and death [20,21]. Prior to development of the hydantoin derived muscle relaxant dantrolene, MH was the most frequent cause of death during anaesthesia [21]. Subsequently, dantrolene was demonstrated to directly inhibit Ca^{2+} fluxes via RyR1 and RyR3, though not RyR2 [22]. The majority of MH cases are associated with point mutations in *RYR1* [20]. To date, more than 100 such mutations have been identified and these are clustered in three regions of the RyR1 protein: N-terminal, central and C-terminal. It has been proposed that intramolecular or inter-subunit interactions between these regions are essential for appropriate gating and that mutations 'unzip' these domains, destabilising the channel, making it hypersensitive to agonists such as Ca^{2+}, cADPr, caffeine and halogenated anaesthetics [23,24]. Certain point mutations, or combinations of them in compound heterozygotes, underlie diseases exhibiting more overt myopathic phenotypes than MH, including central core disease (CCD), multi-minicore disease and fetal ataxia [20]. Patients suffering from CCD display myopathy, hypotonia, delayed motor milestones (developmental timepoints at which specific motor tasks, such as walking, can be performed) and central 'cores' lacking oxidative enzyme activity, running along skeletal muscle fibres. RyR1 mutations associated with CCD cause loss of channel function, in contrast to those resulting in MHS, which promote enhanced sensitivity to channel activators [25]. CCD is present at birth and progresses slowly during development, implying that it is developmental in origin.

Mutations in the *RYR2* gene underlie two forms of stress-induced arrhythmia termed catecholaminergic polymorphic ventricular tachycardia type 1 (CPVT1) and arrhythmogenic right ventricular dysplasia type 2 (AVRD2) [8,26]. Individuals affected by either disease do not display electrocardiogram abnormalities at rest, but can undergo fatal arrhythmias in response to exercise or emotional stress. More than 80 RyR2 mutations underlying such heart disorders have been identified to date and these are clustered in three regions of the protein, analogous to the distribution of MH causing mutations in RyR1. The molecular mechanisms by which RyR2 mutations cause arrhythmia are currently under intensive debate, but include weakened interdomain interactions, enhanced phosphorylation of the channel by protein kinases, altered sensitivity to luminal or cytoplasmic Ca^{2+} concentration and modified interactions with accessory proteins [26].

In addition to the inherited diseases described, RyRs underlie a variety of other disorders. Although it is well established that dysfunction of EC-coupling in heart failure (HF) involves reduction in SR lumenal Ca^{2+} content, participation of RyR2 in this process is uncertain [8,26]. Kobayashi et al. reported that dantrolene corrects faulty interdomain interactions in RyR2 complexes isolated from a ventricular pacing model of HF in dogs. Ventricular cardiomyocytes from these animals display arrhythmogenic delayed afterdepolarisation Ca^{2+} transients, which were abolished by treatment with dantrolene, implying that this muscle relaxant could be used as a therapeutic reagent in HF [28]. However, this is controversial, since dantrolene is reported to be a selective antagonist of RyR1 and RyR3, having no effect on RyR2 gating [22]. Possible resolutions to these conflicting observations include: (1) as Kobayashi et al. propose, RyR2 undergoes structural alterations during HF, such that it becomes accessible to the inhibitory action of dantrolene [27]; (2) the drug is acting on RyR1 or RyR3, known to be present in both rabbit and human heart [28,29]; (3) RyR1/3 play increasingly important roles in cardiomyocytes during the progression of this disease: increased expression of RyR1 in the human myocardium during HF has been reported [29]; and/or (4) dantrolene could exert a positive inotropic effect in HF due to direct inhibition of a RyR1/3-dependent Ca^{2+} 'leak pathway', leading to enhanced SR Ca^{2+} loading and increased Ca^{2+} release via RyR2 during systole (Fig. 1). Such possibilities remain to be explored, but use of dantrolene or related compounds for management of HF is promising.

Involvement of RyRs in disorders of striated muscle has been extensively investigated over the past two decades. However, RyR

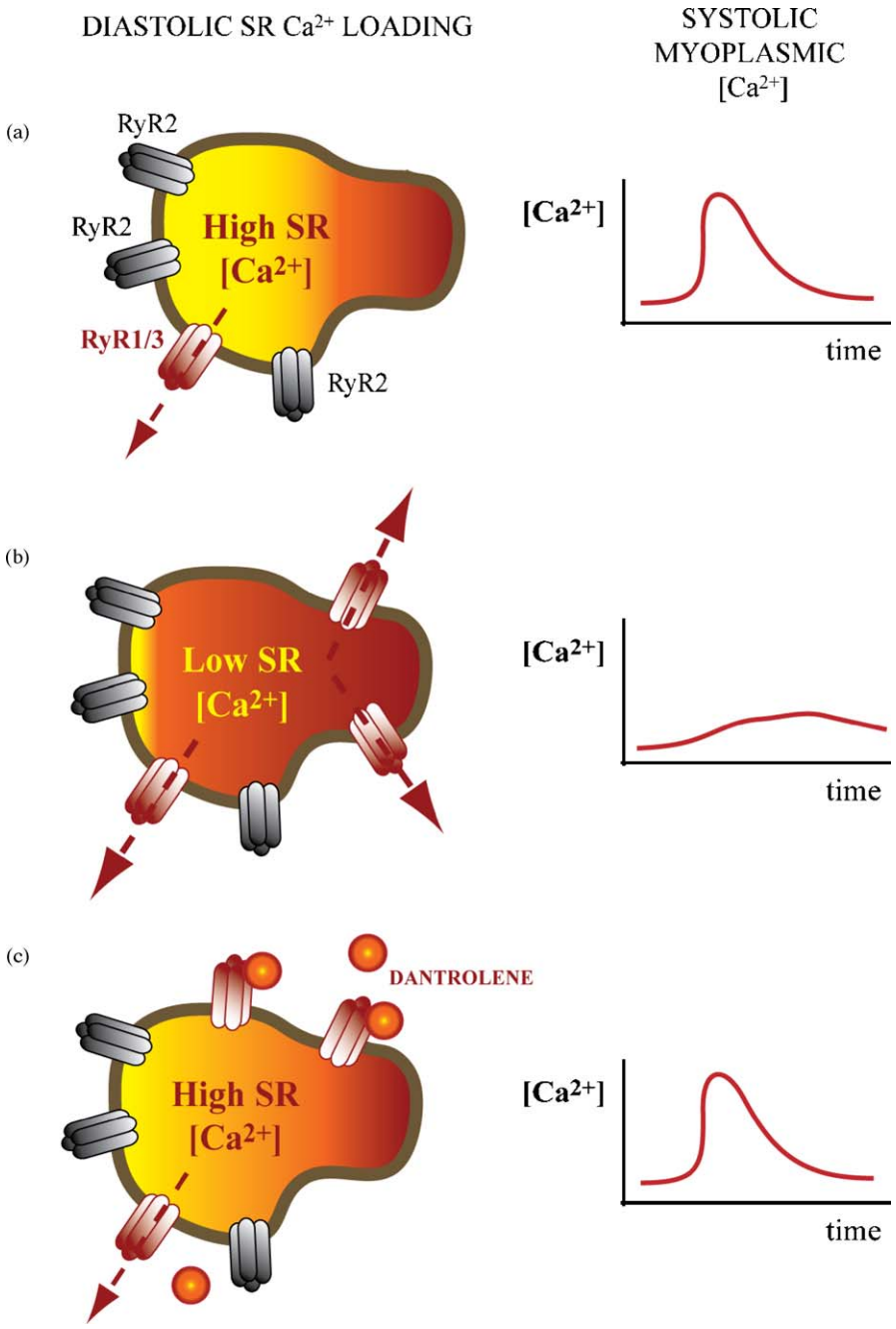

Fig. 1. Potential mechanism by which dantrolene exerts a positive inotropic effect in heart failure. Under normal circumstances, (a) cardiomyocyte SR bears predominantly RyR2 complexes. These channels are usually closed during diastole, permitting accumulation of Ca^{2+} into the SR. This facilitates rapid, high-amplitude myoplasmic Ca^{2+} transients during systole, activating the contractile apparatus to an appropriate level. There is a low level of RyR1/3 expression, which contributes to a small leak of Ca^{2+} from the SR throughout the contractile cycle. During HF, (b) there is an upregulation of RyR1 and/or RyR3, leading to an increased leak of Ca^{2+} from the SR. This causes depletion of Ca^{2+} within the SR lumen, resulting in decreased release of Ca^{2+} through RyR2 channels in response to triggering Ca^{2+} influx during systole. Decreased Ca^{2+} transients lead to reduced contraction that typifies HF. Such RyR1/3-mediated Ca^{2+} release during diastole could also promote arrhythmogenic delayed after-depolarisation currents. Dantrolene exerts a positive inotropic effect in HF, (c) by inhibiting such RyR1/3-mediated Ca^{2+} leak, leading to enhanced Ca^{2+} store loading and Ca^{2+} release via dantrolene-insensitive RyR2 channels during diastole.

complexes are potential therapeutic targets for treatment of a range of other diseases, because of the presence of these channels in non-striated muscle cell types. As indicated in Section 1, defects in RyRs expressed in the nervous system potentially participate in memory defects, epilepsy, depression, pain, neurodegeneration and ischemia [30]. For example, transgenic mice heterologously expressing an *RYR2* mutation (R2474S) that causes CPVT1 in humans display exercise-induced arrhythmias and cardiac sudden death, mimicking the human condition. These transgenic mice also exhibit spontaneous generalised tonic–clonic seizures independently of any effects on the heart, indicating that *RYR2* mutations underlie some forms of epilepsy [31]. It is also postulated that forms of sudden unexplained death in adults [32] and in infants [33] might be a consequence of *RYR2* mutations.

Alterations in RyR expression or function have been implicated in either the progression and or treatment of cancer, owing to the biphasic effects of intracellular Ca^{2+} on cell survival and cell death. For example, a taxol-resistant subclone of the human lung adenocarcinoma A549 displays decreased RyR expression and function relative to the parent cell-line, implying that these

channels participate in the action of certain chemotherapeutic reagents [34]. Similarly, there is a positive correlation between RyR expression levels and tumour grade in breast cancer [35]. By contrast, *in vitro* assays indicate that the RyR1/3 antagonists dantrolene and azumolene induce cell death in low-grade non-Hodgkin's B-lymphoma [36].

In addition to direct roles in disease, RyRs are the site of action for a range of toxins, pesticides and for the side-effects of drugs. Ryanodine itself was originally employed as an agricultural insecticide and several novel compounds with improved selectivity between insect and vertebrate RyRs have been developed commercially, presumably based on differences in primary structure between insect RyRs and their vertebrate counterparts (40–50% amino acid sequence identity) [37]. Like insects, the genomes of clinically or agriculturally important platyhelminth and nematode worms appear to encode a single *RYR* gene. Comparison of the predicted amino acid sequences of RyRs from humans, the mosquito *Anopheles gambiae*, the filarial nematode *Brugia malayia* and the trematode parasite *Schistosoma mansoni* demonstrates that they share 40–50% identity with one another (author's unpublished data). This suggests the potential for development of drugs that selectively target RyRs from economically or medically important parasites and agricultural pests. In support of this, flatworm muscle contraction induced by the RyR agonist caffeine are insensitive to ruthenium red, a 'classical' pharmacological inhibitor of these channels, but are antagonised by ryanodine [38].

Several environmental toxins act on RyRs. For example, myopathy triggered by the organophosphate insecticide fenthion is inhibited by dantrolene [39]. Polychlorinated biphenyls were employed for several industrial applications, until they were banned worldwide prior the mid-1980s owing to their toxicity. However, these compounds are highly stable and persist as environmental toxins, acting on multiple biological targets. One candidate mechanism of this toxicity involves deleterious activation of RyRs, with ortho-substituted polychlorinated biphenyl congeners activating RyR1 and RyR2 at half-maximal effective concentrations in the low micromolar range. Polychlorinated biphenyl congeners that activate RyRs trigger caspase-dependent apoptosis in hippocampal neurons, upregulate RyR1 and RyR2 protein expression in the brain and inhibit certain types of learning in weaning rats [40]. At resting free Ca^{2+} concentrations, non-coplanar 2,2′,3,5′,6-pentachlorobiphenyl enhanced [^{3}H]ryanodine binding (an indirect measure of channel opening) to skeletal muscle SR prepared from pigs bearing the R615C mutation in *RYR1*, but not to SR from wild type animals [41]. This suggests that will be differences in responses between individual humans to environmental toxins and drugs, arising from their distinct *RYR* gene polymorphisms. High-throughput studies will facilitate understanding of the interactions between small molecules such as toxins and various RyR subtypes and polymorphic forms. One such study, assessing the effects of various environmental toxins on [^{3}H]ryanodine binding to SR membranes enriched in either RyR1 or RyR2, highlights the deltamethrin scaffold as a structure from which to develop channel subtype-selective drugs [42].

RyRs are also implicated in the side-effects of several drugs, the archetypal example being the MH-triggering effect of certain anaesthetics in genetically susceptible individuals. Statins are cholestorol-lowering drugs that can exert myopathic side-effects. Such myopathy is associated with upregulation of RyR3, normally a minor subtype in skeletal muscle [43]. Micromolar levels of the phenothiazine antipsychotic drug trifluoperazine directly activate RyR2, thereby contributing to cardiotoxic side-effects of this compound [44]. However, several drugs might also generate adverse effects via interactions with RyR accessory proteins, as described in Section 4.

3. Which drugs act on RyR complexes?

Of the pharmacological reagents known to directly modulate RyR channel activity, only one, dantrolene, is routinely employed for manipulation of these channels under clinical circumstances (Table 1). Limitations of available drugs for therapeutic manipulation of RyR channels include: (1) *low membrane permeability*: RyRs are intracellular targets so drugs must be able to either diffuse

Table 1
Pharmacological reagents that influence RyR gating directly.

Common name	Chemical nature	Effect on channel activity[a]			Concentration range	Clinical or pharmaceutical use	Off-target or side-effects
		RyR1	RyR2	RyR3			
Ryanodine	Alkaloid	+/−	+/−	+/−	nM to mM	Unsuitable.	Toxic.
4-Chloro-*meta*-cresol (CmC)	Chlorinated phenol	+	+	0	μM to mM	Fungicide.	Environmental toxin.
						Preservative.	Activates respiratory burst in neutrophils.
Caffeine (and congeners)	Methylxanthine	+	+	+	mM	Stimulant.	Phosphodiesterase inhibitor.
						Food ingredient.	Adenosine receptor antagonist.
Dantrolene (and congeners)	Hydantoin derivative	−	0	−	μM	Treatment of malignant hyperthermia, muscle spasticity, neuroleptic malignant syndrome and ecstasy intoxication.	Hepatotoxic. Pleural effusion. Central nervous system and gastrointestinal side-effects.
Procaine and tetracaine	Amino ester	−	−	−	μM to mM	Local anaesthetics.	Voltage-gated sodium channel antagonists. NMDAR and 5-HT3R antagonists. Biphasic effects on GABA(A)R.
Ruthenium red	Polycationic dye	−	−	−	nM to μM	Currently none (membrane impermeant).	Modifies TRP cation channels and mitochondrial calcium transporter.

Such substances bind directly to motifs present within RyR monomers, or in the case of ryanodine, to sites that are only formed in the tetrameric state.
[a] For effects on channel gating, '−' indicates a decrease in open probability, '+' signifies an increase and '0' signifies no effect.

across membranes, or be imported by cellular transporters, in order to act on these channel complexes; (2) *low solubility in aqueous solutions* for membrane-permeant drugs, such as dantrolene; (3) *lack of specificity*: for example, caffeine and local anaesthetics act on multiple systems; (4) *poor selectivity* between RyR subtypes; no available drug acts on just one RyR isoform; (5) *low potency*: for example, caffeine stimulates RyR gating in the millimolar range; (6) *toxicity via direct action on RyRs*: for example, effects of ryanodine on RyR gating are complex, displaying both biphasic dose–response relationships (activating then inhibiting channel opening) and 'use-dependence' (only binding to open channels); and (7) *indirect toxicity*: for example, dantrolene metabolites might be hepatotoxic via a mechanism independent of RyR gating: however, this hepatic toxicity is a matter on controversy, since it is not detectable in isolated hepatocytes [45].

Consequently, future therapeutic interventions targeting RyRs could include: (1) discovery of novel drugs acting directly, selectively and efficaciously on single RyR isoforms; (2) molecular genetic approaches, which might be of limited scope for RyR associated pathologies that display developmental aetiologies; and (3) development of drugs targeting tissue-specific RyR interacting proteins. The remainder of this commentary focuses on possible strategies based on manipulation of RyR function via their interacting proteins, or via the cellular mechanisms that regulate the localisation or expression levels of these channel complexes.

4. RyR accessory proteins

The functional core of RyRs is a tetramer: these proteins do not act as channels in the monomeric state. Such complexes do not operate in isolation. In 1999, Mackrill first suggested that '...the functional properties of ... RyRs within particular cells and subcellular domains are 'customised' by the accessory proteins present' [19]. This concept has been expanded to generate themes such as that of cells expressing a specific 'Ca^{2+}-toolkit' [18] that suits their function or underlies their dysfunction in disease; and the strategy of developing therapeutic regimes targeting RyR accessory proteins [46]. Such protein–protein interactions enable RyRs to integrate a wide range of intracellular signals, including the second messengers Ca^{2+}, cyclic AMP, cyclic GMP, nitric oxide and reactive oxygen species [19]. RyR gating is modulated by a range of protein kinases and phosphatases, highlighting these enzymes as potential drug targets [8,26]. As a consequence of their subcellular localisation, RyRs are able to communicate with interacting proteins and other biomolecules present in multiple subcompartments, namely the ER/SR lumen and membrane, the cytosol and the plasma membrane (Fig. 2). As a result, there is considerable scope for modification of RyR gating via drugs acting on their accessory proteins. It is unlikely that all RyR interacting proteins have been identified to date: a review of the literature indicates that at least 12 novel RyR interacting proteins have been identified over the last decade. This is not particularly unexpected, given the size and highly solute accessible structure of these complexes, as

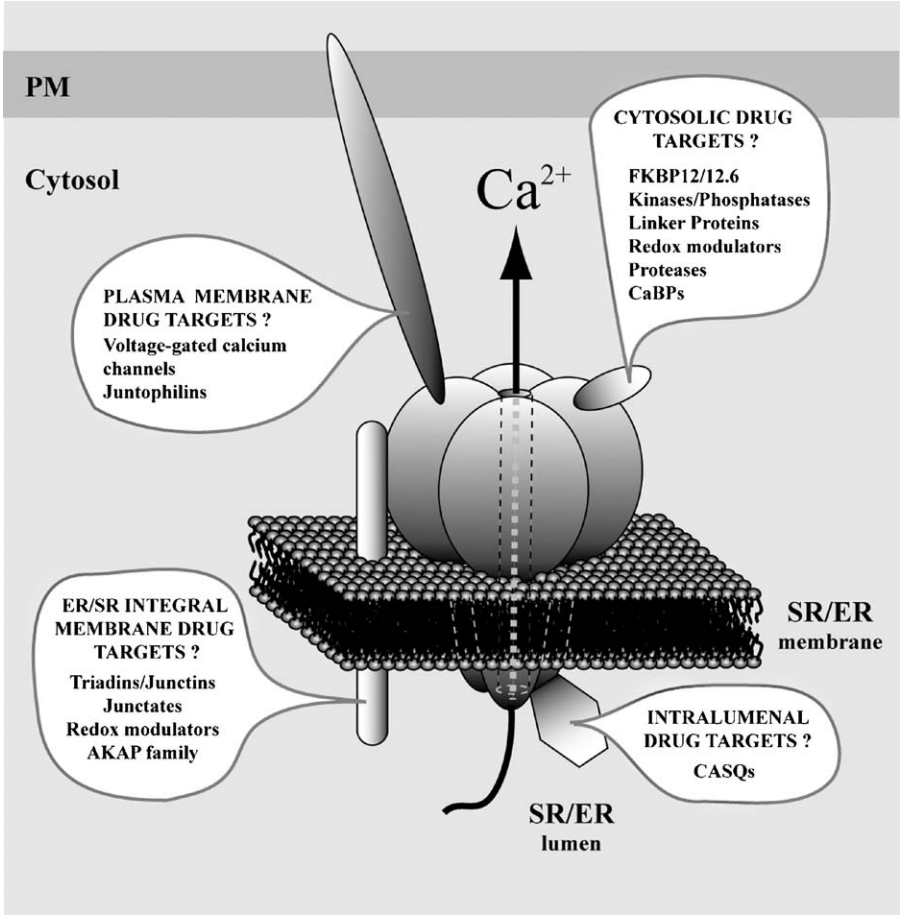

Fig. 2. The subcellular localisation of RyRs enables them to interact with components of four different cellular compartments. Since most RyR complexes are located in the SR or ER, they can communicate with four distinct compartments: the SR/ER lumen, the SR/ER membrane, the cytoplasm and the inner face of the plasma membrane (PM). This property enables RyRs to integrate a wide variety of intracellular signals, including those from accessory proteins present within each of these domains. RyR interacting proteins present within each of these subcellular compartments represent targets for the development of therapeutic drugs for treatment of diseases associated with dysregulated gating of these channels.

well as the potential of different accessory proteins interacting with the same sites within these channel proteins. For example, Ca^{2+} occupied forms of calmodulin and S100A1 interact with overlapping regions of a site conserved in both RyR1 and RyR2, but exert opposing effects on channel gating [47].

Certain RyR accessory proteins, such as calmodulin and the 12/12.6 kDa FK506-binding proteins (FKBP12, FKBP12.6; members of the FKBP family 'rebranded' as 'calstabin-1 and -2' by a few authors), are ubiquitously expressed and could be considered as obligatory components of RyR complexes. Disruption of interactions between RyR2 and FKBP12.6, possibly by cyclic AMP-dependent kinase hyperphosphorylation of the channel protein, is one mechanism proposed to underlie the pathology of HF [8,26]. Recently, the therapeutic potential of this interaction has been demonstrated in transgenic mice overexpressing FKBP12.6 specifically in heart muscle: cardiomyocytes from these animals display a reduction isoproterenol-induced tachycardia relative to wild type cells [48].

Other RyR accessory proteins display highly restricted tissue expression patterns: for example, calsequestrins (CASQs) are SR/ER intralumenal Ca^{2+}-binding proteins only present at high abundance in skeletal muscle, heart, certain smooth muscles and in cerebellar Purkinje neurons [49]. However, RyRs are expressed throughout the nervous system, as well as in many 'non-excitable' cell types [10,11], indicating that CASQs are not required for normal RyR function in these cells. However, there is considerable evidence to support pivotal roles of tissue-specific accessory proteins, such as CASQs, in RyR function. For example, mutations in human *CASQ2*, the predominant form in heart, underlie arrhythmias that clinically resemble those caused by certain *RYR2* mutations [50]. Similarly, certain mutations in $Ca_v1.1$, the pore-forming subunit of skeletal muscle voltage-gated calcium channels that physically interacts with RyR1 to facilitate skeletal muscle EC-coupling, are associated with MH phenotypes that are clinically indistinguishable from those caused by *RYR1* mutations [19].

To date, only a limited number of drugs have been reported to act by modifying interactions between RyRs and their accessory proteins (Table 2). FK506 and rapamycin are macrolide immunosuppressants that exert their effects via distinct mechanisms. In addition to their immunosuppressive effects, both macrolides disrupt interactions between RyRs and FKBP12/12.6, enhancing subconductance states, thereby generating 'leaky' channels [26]. The 1,4-benzothiazepine K201 (also known as JTV519) and certain congeners stabilise the interaction between RyR2 and FKBP12.6, thereby enhancing 'fully open' and 'fully closed' states of this channel. Consequently, K201 could be considered as functionally antagonistic to the effects of FK506 or rapamycin on FKBP-RyR interactions. K201 has the potential for treatment of HF, since it corrects defective RyR channel gating [51] and FKBP12.6 binding [52] in a ventricular pacing animal model of this disease. K201 might also stabilise RyR2 complexes present in medullary collecting duct cells of the kidney, thereby enhancing glomerular filtration rate, natriuresis, diuresis and renal vasodilation, contributing to the cardioprotective effects of this drug [53]. However, the actions of K201 are not selective. This compound also stabilises the binding of FKBP12 to RyR1 [54] and inhibits the activity of SR/ER Ca^{2+}-ATPases that pump Ca^{2+} into the SR lumen [55]. Despite this, there are no reports in the literature of K201 influencing the activities of other targets of FK506 or rapamycin, such as calcineurin, RAFT or mTOR.

Other therapies might correct dysfunctional interactions between FKBP12/12.6 and RyRs via mechanisms that have not been well characterised. The heart rate reducing drug ivabradine acts by inhibition of I_f current in pacemaker cells of the sinoatrial node. Treatment of a rabbit model of reperfusion injury with this drug also results in an upregulation of FKBP12/12.6 protein in the heart, without altering expression levels of other key EC-coupling proteins, such as RyR2. It was proposed that ivabradine improves hemodynamic parameters in this model of heart injury by a dual mechanism, involving both stabilisation of the FKBP12/12.6-RyR2 interaction and I_f current inhibition [56]. Endothelin receptor antagonists are reported to have beneficial effects on various cardiomyopathic states. For example, CPU0213 ameliorates cardiac insufficiency in a rat model of diabetes, associated with increases heart expression of FKBP12.6, SERCA2a Ca^{2+} ATPase and its modulatory protein phospholamban [57]. Although the mechanisms by which K201, ivabradine and endothelin antagonists modulate the function or expression of RyR accessory proteins are unclear, these examples illustrate that polytherapeutic strategies, using several drugs or one 'promiscuous' drug to influence multiple cellular targets, could be highly beneficial in the treatment of heart diseases.

Certain drugs modify the properties of CASQs. When SR lumenal free Ca^{2+} concentrations are in the millimolar range, CASQ exists as a multimeric polymer that acts as both a Ca^{2+} reservoir and an enhancer of RyR gating, effectively acting as a sensor of the loading status of the Ca^{2+} store. At lower concentrations of this ion, CASQ exists in a monomeric state that interacts with additional SR proteins known as triadins and junctins, rather than with RyRs [8]. Multiple classes of hydrophobic drug, including doxorubicin, other anthracyclines, phenothiazines and tricyclic antidepressants, exert cardiotoxic side-effects such as promotion of arrhythmias or HF. However, such adverse effects are tolerated because of the clinical usefulness of these compounds. Many of these drugs directly bind

Table 2
Pharmacological reagents that influence RyR accessory protein interactions.

Common name of drug(s)	Effect on RyR interactions	Concentration range	Clinical or pharmaceutical use	Comments
FK506 (tacrolimus)	Disrupts RyR1/3-FKBP12/12.6 and RyR2–FKBP12.6 interaction, causing 'leaky' channels	nM to μM	Immunosuppressant.	FKBP-FK506 complexes inhibit calcineurin.
Rapamycin (sirolimus)	Disrupts RyR1/3-FKBP12/12.6 and RyR2–FKBP12.6 interaction, causing 'leaky' channels	nM to μM	Immunosuppressant.	FKBP-rapamycin complexes bind FKBP-rapamycin associated protein (FRAP, RAFT or mTOR).
K201 (JTV519) and congeners	Stabilise RyR–FKBP12/12.6 interactions	nM to μM	Candidate cardioprotective reagents.	Antagonist of multiple ion channel subtypes. Inhibits SERCA pumps.
Doxorubicin (and other anthracyclines, phenothiazines, or tricyclic antidepressants)	Decrease binding of calcium ions to CSQs. Disrupt interactions between RyRs and CSQs.	μM	Chemotherapeutics (and many other classes of drug, including antidepressants).	Candidate mechanism by which many classes of drug exert myopathic or cardiotoxic side-effects.

to CASQ2 with micromolar affinities, decreasing its Ca^{2+} binding. Park et al. envisage that analogues of such drugs could be rationally redesigned in order to negate toxicity resulting from their effects on CASQs [58]. It is tempting to speculate that drugs designed to enhance CASQ Ca^{2+} binding and polymerisation could be developed for treatment of HF or certain arrhythmias. Alternatively, therapeutic reagents for modulation of interactions between CASQ and triadin or junctin could also be used for management of such diseases. Additional components of RyR–CASQ complexes, such as the histidine-rich Ca^{2+}-binding protein, triadin and junctin, represent candidate drug targets, particularly for treatment of arrhythmias, ischemic damage or HF. For example, cardiac-specific overexpression of histidine-rich Ca^{2+}-binding protein, a triadin-interacting partner of the SR lumen, is protective in a mouse model of ischemia/reperfusion injury [59].

Drugs designed to modulate RyR gating via tissue-specific accessory proteins would ideally possess the following characteristics: (1) would be membrane-permeant (hydrophobic or capable of being imported by cellular transport mechanisms), since RyRs are intracellular; (2) would selectively interact with tissue-specific accessory proteins; and (3) would display a high benefit/risk ratio. Such therapeutic compounds await development.

5. Regulation of RyR expression and subcellular localisation in disease

Certain proteins modulate RyR function via mechanisms distinct from acting as permanent, stoichiometrically associated components of these channel complexes: such proteins also represent targets for disease- or tissue-specific modulation of RyRs. For example, selenoprotein N1 is an ER/SR membrane protein, mutations in which can result in multi-minicore disease, a skeletal myopathy that also results from certain *RYR1* mutations [60]. This protein binds to RyR complexes and modulates their gating by acting as a redox sensor [61]. However, expression of SEPN1 protein declines rapidly following birth, hinting that it has key roles during development, rather than in adult animals [60]. Indeed, zebrafish embryos with reduced expression of either SEPN1 or RyR3 display a loss of left–right asymmetry in the cytoplasmic Ca^{2+} levels in cells of the Kupffer's Vesicle, with subsequent abnormalities in skeletal muscle development [61].

Currently, data on control of *RYR* gene expression is scant, although a variety of extracellular cues have been reported to regulate levels of transcription of these genes [1,10]. Furthermore, the cardioprotective drug dexrazoxane is known to reduce downregulation of RyR2 mRNA in the heart in anthracycline-induced cardiomyopathy [62]. Understanding the transcriptional and translational apparatus controlling expression of *RYR* genes is of medical importance, since these mechanisms also represent suitable therapeutic targets. This is particularly pertinent, given the large differences in expression of *RYR* gene expression observed between different tissues and because of the reported alterations in transcription of these channels during the progression of certain diseases, such as HF [29]. Similar rationales apply to understanding the transcriptional control of expression of RyR interacting proteins.

Analyses of the promoter of the *RYR1* gene indicates that expression requires specificity protein-1 (Sp1), myocyte enhancing factor-2 and at least two unidentified transcription factors; whereas tissue-specificity is mediated by transcriptional repression in non-muscle cells, which requires sequences within the first intron [63]. *RYR1* gene expression is also subject to epigenetic regulation. Certain pedigrees of patients suffering core myopathies display monoallelic expression of mutated *RYR1* genes. This monoallelic expression is tissue-specific, developmentally regulated and is likely to be mediated via an imprinting mechanism, involving hypermethylation of one *RYR1* allele, thereby unveiling a recessive mutation [64].

Analyses of the *RYR2* promoter indicate that expression requires binding of Sp1 and at least two unidentified transcription factors; whereas tissue-specificity is mediated by sequences between −209 and −90 bases of the transcription start site [65]. Certain transcription factors controlling *RYR2* gene expression have been identified. In mammals, circadian rhythms are regulated via an endogenous rhythm generator within the suprachaismatic nucleus of the brain. RyR2 participates in resetting this rhythm in response to light in the early night, by releasing Ca^{2+} which results in phase delays. The transcriptional activator BMALI, a downstream component of this molecular clock, stimulates expression of *RYR2*, whereas mCRY1, a downstream transcriptional inhibitor, represses transcription of this calcium channel gene. Consequently, transcription factor components of the mammalian molecular clock regulate levels of RyR2 protein, thereby controlling the input from early night light stimulation [66].

Transgenic mice lacking functional copies of the transcription factor Nkx2-5 display conduction defects and dilated cardiomyopathy, along with reduced expression of various proteins involved in ECC, including RyR2 [67]. Chronic sympathetic stimulation is a key component in the aetiology of HF and involves β-adrenergic receptor stimulated alterations in gene expression, mediated via the transcription factors cAMP response element binding protein (CREB) and cAMP response element modulator (CREM). Inactivation of *CREM* in a mouse model of HF (heart directed overexpression of the β1-adrenergic receptor) protected against hypertrophy, left ventricular dysfunction and fibrosis. *CREM* knockout in transgenic mice enhanced expression of various cardiac muscle proteins, including RyR2 [68].

All *RYR* genes undergo posttranscriptional processing by mRNA splicing, to increase structural and functional specialisation of these channels. For example, RyR3 is extensively spliced in a tissue-specific and developmentally regulated manner. One smooth muscle-specific RyR3 splice variant, lacking a predicted transmembrane helix, acts as a tissue-specific dominant negative regulator of RyR3 channels [69]. Heart-specific ablation of the splicing factor SC35 leads to dilated cardiomyopathy, defective EC-coupling and decreased RyR2 expression in transgenic mice. Since viral myocarditis can also decrease SC35 expression, Ding et al. proposed that alteration splicing patterns could underlie the cardiac pathology caused by such pathogens [70]. Expression of two distinct RyR2 splice-variants in HL-1 cardiomyocytes indicated a potential mechanism by which defects in mRNA splicing mechanisms could result in heart disease: one variant protected cells from prolonged elevations in cytoplasmic Ca^{2+} and apoptosis triggered by caffeine, whereas the other splice form did not [71].

Cellular trafficking mechanisms influence RyR function by determining the subcellular distributions of these channels. Particular splice-variants [71] or mutations [72] in RyR2 alter the distribution of these channel complexes, such that an increased proportion resides in the Golgi apparatus. In a spontaneously hypertensive rat model of HF, there is an increased proportion of RyR2 channels that do not reside in close proximity to voltage-gated calcium channels, owing to restructuring of the t-tubular system. It is proposed that such 'orphaned' RyR2 channels, which are not in direct communication with the t-tubular system, lead to unsynchronised Ca^{2+} release and potentially to arrhythmia [73].

In centronuclear myopathy, also known as myotubular myopathy, there is a remodelling of the SR around central nuclei (normally, nuclei are peripherally located) within skeletal muscle fibres. Five causative genes have been reported to underlie this disease: (1) myotubularin and (2) myotubularin-related protein 14 (MTMR14), both phosphatidylinositol phosphate phosphatases, potentially involved in membrane biogenesis; (3) BIN1, or amphysin, a protein

that generates membrane curvature; (4) RyR1; and (5) dynamin-2, a molecular motor protein involved in membrane transport [74]. Transgenic mice lacking MTMR14 display muscle fatigue, resulting from direct activation of RyRs by accumulated phosphatidylinositol phosphate substrates of this enzyme [75]. It is also likely that all of the causative genes in myotubular dystrophy play roles in the assembly of specialised membrane junctions, dysfunction of which would result in defective EC-coupling.

6. Future directions

Cellular proteins modulating gating, abundance or subcellular distribution of RyR complexes represent therapeutic targets of considerable potential. However, there are many issues surrounding the biology and pharmacology of these channels that must be resolved before this potential is fulfilled: (1) there is a need for systematic identification of RyR accessory proteins, and how these differ during development, between distinct tissues and during progression of disease; (2) information on how transcription of different *RYR* genes is regulated is scant: such detail is required in order to permit pharmacological manipulation of the levels of these channels; (3) similarly, understanding of how RyR subcellular distribution is controlled is limited; and (4) molecular mechanisms of the interactions between RyRs and their accessory proteins, and between RyRs and pharmacological reagents, is continually being resolved in greater detail, but structural analyses are hampered by the high molecular weight and hydrophobic domains within these channel complexes. To date, details of the relationships between RyR structure and function have been resolved by analyses of smaller synthetic fragments [46,47], by generation of chimeric and point-mutated proteins, or by cryoelectron microscopy in combination with image reconstruction of recombinant channels bearing fluorescent proteins inserted within specific regions of the primary structures. These investigations of RyR properties will require considerable investments of research effort in order to generate clinically applied outcomes. Such outputs will include rationally designed drugs that enhance or disrupt RyR accessory protein interactions to alter gating of these channels. It is envisaged that progress in any or all of these areas will lead to the development of novel therapeutic strategies for the treatment of RyR associated diseases.

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