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Differential Ca^{2+} sensitivity of RyR2 mutations reveals distinct mechanisms of channel dysfunction in sudden cardiac death ☆

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Abstract

Arrhythmogenic point mutations in RyR2 result in abnormal Ca^{2+} release following cardiac stimulation, leading to sudden cardiac death (SCD). Recently, we have demonstrated that significant functional differences exist between SCD-linked RyR2 mutations. Here, we investigated the molecular basis of this heterogeneity and determined the sensitivity of mutant RyR2 channels to cytoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) in living cells. Using streptolysin-O permeabilised human embryonic kidney cells, $[Ca^{2+}]_c$ was clamped in cells expressing GFP-tagged wild-type (WT) or SCD-linked RyR2 mutants ($L^{433}P$, $N^{2386}I$, and $R^{176}Q/T^{2504}M$). Although resting $[Ca^{2+}]_c$ was comparable in all cells, RyR2 mutants were characterised by a profound loss of Ca^{2+}-dependent inhibition following caffeine stimulation when compared with WT channels. The ER Ca^{2+} store was not perturbed in these experiments. Our findings support the hypothesis that SCD-linked mutational loci may be an important mechanistic determinant of RyR2 dysfunction and indicate that there is unlikely to be a unifying mechanism for channel dysfunction in SCD.

Keywords: Ryanodine receptor; Mutations; Ca^{2+} release channel; Dysfunction; Arrhythmia; Sudden cardiac death

Ca^{2+} release through ryanodine receptors (RyR) regulates the excitation–contraction (E–C) coupling process in cardiac muscle [1]. Arrhythmogenic point mutations in cardiac RyR (RyR2) are proposed to mediate abnormal Ca^{2+} release following cardiac stimulation, frequently resulting in sudden cardiac death (SCD) [2]. To date, 35 mutations in RyR2 have been associated with catecholaminergic polymorphic ventricular tachycardia (CPVT) and arrhythmogenic right ventricular dysplasia type 2 (ARVD2), cardiac pathologies characterised by delayed after-depolarisations (DADs) and Ca^{2+} overload following physical or emotional stress, which lead to VT and sudden death [3–10]. Intriguingly, despite the vast size of RyR2 (~5000 aa), these reported mutations cluster in three highly conserved regions of RyR2 (amino (N-) terminal, 17%; central domain, 26%; and carboxyl (C-) terminal, 55%; where percentages represent their relative distribution) and are associated with varying clinical severity [4,7].

So far, functional characterisation of 11 SCD-linked RyR2 mutations following their heterologous expression in a variety of cellular systems has yielded important insights into the mechanisms of RyR2 dysfunction [11–16]. However, the precise role of these mechanisms in causing abnormal Ca^{2+} release through RyR2 remains highly controversial. SCD-linked mutations occurring in the central and C-terminal domains have been shown to decrease the affinity of RyR2 for

☆ *Abbreviations:* SCD, sudden cardiac death; $[Ca^{2+}]_c$, cytoplasmic $[Ca^{2+}]$; ER, endoplasmic reticulum; RyR2, cardiac ryanodine receptor; WT, wild-type; GFP, green fluorescent protein; SLO, streptolysin-O; HEK, human embryonic kidney cells; CPVT, catecholaminergic polymorphic ventricular tachycardia; ARVD2, arrhythmogenic right ventricular dysplasia type 2; DAD, delayed after-depolarisation; SERCA, sarco-endoplasmic reticulum Ca^{2+}-dependent ATPase; CRT, calreticulin; CSQ, calsequestrin; TG, thapsigargin.

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FKBP12.6, a key modulator of RyR2 function [15]. The selective dissociation of FKBP12.6 from RyR2 following PKA-mediated channel phosphorylation has been proposed as a causative event in the pathogenesis of heart failure [17], and the loss of FKBP12.6 from the RyR2 complex was suggested to directly increase the arrhythmogenic propensity in SCD-susceptible individuals [12,15]. However, the interaction between FKBP12.6, and central and C-terminal domain SCD-linked mutations was indistinguishable to that determined with WT RyR2 in resting and stimulated cardiomyocytes [11]. Recently, the precise molecular mechanisms proposed to underlie FKBP12.6:RyR2 dissociation have been brought into question [18–20]. Furthermore, although FKBP12.6 binding to RyR2 may be entirely dependent on the conformational architecture of the channel, it is difficult to envisage how all reported mutations in physically and functionally distinct regions of RyR2 would lead to pathogenic dissociation of the RyR2:FKBP12.6 complex. Recently, it has been shown that three C-terminal RyR2 mutations sensitised RyR2 to activation by the ER Ca^{2+} store [14], consistent with the role of altered lumenal Ca^{2+} environment in arrhythmia [21] and thereby representing an FKBP12.6-independent mechanism of channel dysfunction in arrhythmogenesis. We hypothesise that the complexity of RyR2 structure–function and the number of reported mutations precludes a single unifying mechanism of RyR2 dysfunction. This hypothesis was reinforced by the characterisation of a desensitised RyR2 mutation ($L^{433}P$), a finding that also challenged the perception that all RyR2-linked arrhythmogenic disorders result exclusively from 'gain-of-function' mutation [16].

It has been proposed that altered sensitivity of mutant RyR2 to cytoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) may underlie SCD in arrhythmia-susceptible individuals [4]. In the present work, we investigated the sensitivity of functionally heterogeneous ARVD2-linked N-terminal and central domain mutants ($L^{433}P$, $N^{2386}I$, and $R^{176}Q/T^{2504}M$) to $[Ca^{2+}]_c$ in a permeabilised, living cell context. Our findings reveal that N- and central domain mutations are associated with profoundly altered channel sensitivity to $[Ca^{2+}]_c$ that markedly perturbs the Ca^{2+}-dependent inhibition of the channel. Our data provide further evidence to support the hypothesis that RyR2-dependent arrhythmia arises from complex mechanistic bases.

Materials and methods

All cell culture reagents and plasticware were obtained from Invitrogen (Paisley, UK). All electrophoresis reagents and equipment were from Bio-Rad. All other chemicals and reagents were from Sigma, except where indicated. The anti-green fluorescent protein (GFP) monoclonal antibody (clone B-2) was obtained from Santa Cruz Biotechnology (CA, USA) and was used at 1:5000 dilution. The sarco/endoplasmic reticulum Ca^{2+} ATPase isoform 2 (SERCA2) monoclonal antibody (clone MA3-919) was obtained from Affinity Bioreagents (CA, USA) and was used at a 1:10,000 dilution. Rabbit polyclonal antibodies against calsequestrin (CSQ) and calreticulin (CRT) were obtained from Abcam (Cambridge, UK; used at 1:2500 dilution) and D.H. Llewellyn (Cardiff University; used at 1:1000 dilution) [22], respectively.

Cellular permeabilisation using streptolysin-O and manipulation of $[Ca^{2+}]_c$. Human embryonic kidney (HEK) cells expressing GFP-tagged wild-type (WT) or SCD-linked mutant human recombinant RyR2 were generated and maintained as previously described [16]. Cells were incubated with fluo3-AM (10 μM, in DMSO containing 20% (w/v) Pluronic F127) (Biotium, CA, USA) in Dulbecco's modified Eagle's medium (DMEM) without serum for 90 min at 37 °C. Cells were then permeabilised in Krebs–Ringer–Hepes buffer (KRH; 120 mM NaCl, 25 mM Hepes, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 5.5 mM glucose; pH 7.4) containing fluo3-AM (10 μM) and streptolysin-O (SLO). Following titration of SLO permeabilisation conditions [23,24], we determined that an optimal SLO concentration of 100 U ~50 μg protein/200 μl/1.5×10^5 cells/22 mm^2 for 30 min resulted in specific plasma membrane permeabilisation of ~80% of the cell population (as determined by trypan blue infiltration), with cells retaining viability and fluo3 fluorescence (data not shown).

Following SLO permeabilisation, cells were immediately washed in solution (100 mM KCl, 20 mM Hepes, and 1 mM $MgCl_2$; pH 7.4), followed by incubation for 5 min in this solution containing known $[Ca^{2+}]$ (0.1 nM–61 μM) to clamp $[Ca^{2+}]_c$. Known $[Ca^{2+}]$ in these solutions were obtained using different $CaCO_3$:EGTA ratios calculated using REACT II software (Cybersolutions; http://www.cyber-sol.co.uk). Cells were equilibrated in these solutions for no more than 5 min, in order to maintain ER Ca^{2+} load status.

Intracellular Ca^{2+} imaging. Fluo3 loaded cells were imaged using a confocal microscope (Leica RS2; Leica, Heidelberg, Germany) controlled by Leica software. The Ca^{2+}-dependent fluorescence of fluo3 was calibrated in SLO permeabilised cells as described [24]. An apparent K_d ($K_{d,app}$) of 733 nM was generated using GraphPad Prism software. $[Ca^{2+}]_c$ was calculated from fluorescent data using: $[Ca^{2+}] = K_{d,app}(F - F_{min})/(F_{max} - F)$ where F represents fluorescence, and F_{min} and F_{max} represent the minimum and maximum fluorescent signals determined following the addition of EGTA (20 mM) and ionomycin (2 μM), respectively. Cells expressing recombinant WT or mutant RyR2 were identified by the addition of 0.5 mM caffeine, followed by wash-out and complete solution exchange with buffer containing known $[Ca^{2+}]_c$.

At each clamped $[Ca^{2+}]_c$, Ca^{2+} release via RyR2 was triggered following the addition of a bolus of caffeine (10 mM). Each addition of caffeine was to separate cell populations to negate the effects of sequential caffeine addition on cellular Ca^{2+} handling. ER Ca^{2+} load was estimated from peak Ca^{2+} release following the addition of thapsigargin (TG, 5 μM) to cells [16,25]. Data were acquired from regions of interest representing global Ca^{2+} environments (typically approximately 50 μm^2), and analysed using Leica confocal and Origin 7 software. Statistical analysis was performed using unpaired Student's t test and one-way ANOVA.

Immunoblotting analysis of recombinant RyR2 and endogenous Ca^{2+} handling proteins. The relative expression of recombinant WT and mutant GFP-tagged RyR2 protein was determined using an anti-GFP monoclonal antibody [16]. The endogenous levels of ER resident proteins (SERCA, CRT, and CSQ) in cells expressing WT and mutant RyR2 were determined following SDS–PAGE separation of post-nuclear supernatant fractions obtained from cells (100–250 μg) and subsequent immunoblot analysis [25]. Untransfected HEK cells or rabbit cardiac microsomes (50 μg) [26] were used as controls. Densitometric analysis of immunoblots was carried out using a densitometric scanner (GS700, Bio-Rad) and Quantity One software (Bio-Rad).

Results

We investigated the effect of SCD-linked mutations on the Ca^{2+} dependence of caffeine-triggered Ca^{2+} release in living HEK cells. Recombinant WT RyR2 exhibited a biphasic curve with maximal caffeine activation occurring at $[Ca^{2+}]_c$ of 380 nM. As $[Ca^{2+}]_c$ increased >380 nM, caffeine activation of RyR2 was progressively inhibited, returning to almost complete inhibition at $[Ca^{2+}]_c \sim 10$ μM. The values of $[Ca^{2+}]_c$ for half-maximal channel activation (EC_{50}) and inhibition (IC_{50}) are given in Table 1. The caffeine-triggered activation of RyR2 in our permeabilised cell system is in close agreement with the characterisation of the Ca^{2+} sensitivity of caffeine activation of recombinant RyR2 in planar lipid bilayers [27], demonstrating that RyR2 remains fully functional following cellular permeabilisation. The $N^{2386}I$ mutation, occurring in the central domain of RyR2, also exhibited biphasic caffeine activation/inhibition profile (Fig. 1B) characterised by equivalent EC_{50} and IC_{50} values to

Table 1
Ca^{2+} dependence of SCD-linked RyR2 mutations

RyR2	EC_{50} (nM)	IC_{50} (μM)
WT	42 ± 2	3.3 ± 0.6
$N^{2386}I$	45 ± 26	1.3 ± 1.3
$L^{433}P$	39 ± 9	24 ± 6.6*
$R^{176}Q/T^{2504}M$	146 ± 117	ND

$[Ca^{2+}]_c$ required for half-maximal activation and inhibition (EC_{50} and IC_{50}, respectively) of WT and mutant RyR2 in response to caffeine (10 mM) was calculated following regression analysis of data shown in Fig. 1. Data are given as means ± SE. ND represents not determined.
* $p < 0.01$.

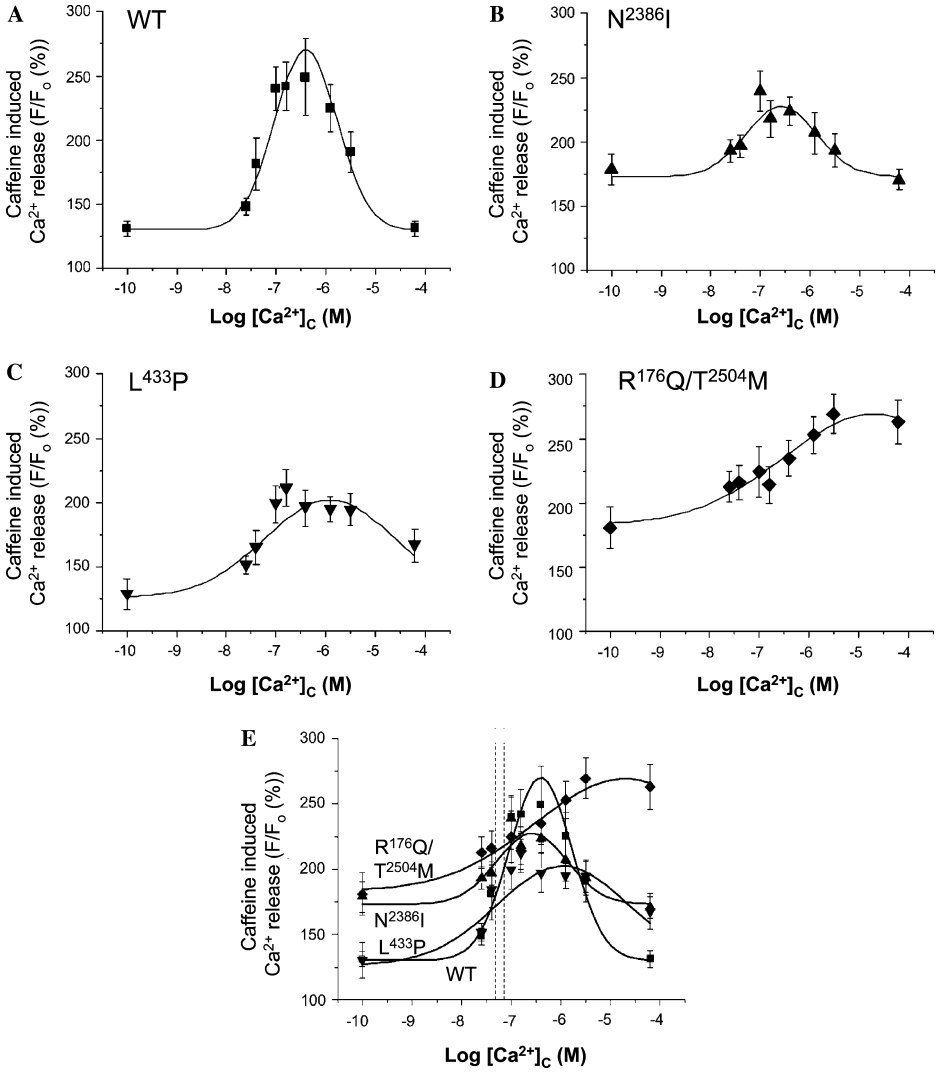

Fig. 1. The Ca^{2+} dependence of caffeine activation of RyR2 mutants in living cells. The activation of WT RyR2 (A), and SCD-linked mutants $N^{2386}I$ (B), $L^{433}P$ (C), and $R^{176}Q/T^{2504}M$ (D) following addition of caffeine (10 mM) to SLO-permeabilised cells at clamped $[Ca^{2+}]_c$ was determined. The graphs are overlaid in (E). The vertical dashed bars indicate the range of resting $[Ca^{2+}]_c$ (~50–90 nM, i.e., log $[Ca^{2+}]_c \sim -7.1$ to -7.3) determined in intact (non-permeabilised) cells. Caffeine-induced Ca^{2+} release was determined as the peak change in fluorescence following caffeine addition (F) relative to resting fluorescent signals (F_o) and is expressed as a percentage. Data are given as means ± SE ($n = 4$ experiments, >15 cells analysed per experiment).

those obtained for WT RyR2 (Table 1). However, these values mask the finding that this mutation profoundly perturbed the Ca^{2+} sensitivity of the channel. Strikingly, the $N^{2386}I$ channel exhibited marked caffeine activation (>160% activation when compared with non-stimulated cells) at a wide range of imposed $[Ca^{2+}]_c$ (0.1 nM–61 μM), indicating that this mutation resulted in a partial loss of Ca^{2+}-dependent channel regulation. Despite this apparent lack of Ca^{2+}-dependent channel modulation following caffeine stimulation, $N^{2386}I$, in addition to the other mutations characterised in this study, does not condition resting cells to elevated $[Ca^{2+}]_c$ in non-stimulated intact cells (WT, $80\,nM \pm 13$; $N^{2386}I$, 65 ± 11 nM; $L^{433}P$, 86 ± 13 nM; $R^{176}Q/T^{2504}M$ 51 ± 13 nM; and $R^{176}Q$ 77 ± 15 nM ($n = 5$, >12 cells per experiment)) strongly suggesting appropriate RyR2 mutant channel regulation in resting cells. $L^{433}P$, a mutation previously determined to exhibit Ca^{2+} release of a magnitude similar to that of WT RyR2, and thus the only non 'gain-of-function' RyR2 mutation so far reported, exhibited a similar Ca^{2+}-dependent activation profile to WT RyR2, but we determined markedly desensitised Ca^{2+}-dependent inhibition (Fig. 1C and Table 1). Like $N^{2386}I$, the 'double mutation' $R^{176}Q/T^{2504}M$, was characterised by a similar activation profile to WT RyR2 (Table 1) that masked a significant Ca^{2+}-independence of caffeine activation of the channel. However, unlike $N^{2386}I$, the $R^{176}Q/T^{2504}M$ mutation exhibited a complete loss of Ca^{2+}-dependent inhibition of Ca^{2+} release following caffeine stimulation (Fig. 1D and Table 1).

We have previously reported that following heterologous expression in HEK cells, $N^{2386}I$ and $R^{176}Q/T^{2504}M$ represent 'gain of function' mutations, whereas $L^{433}P$ exhibited marked desensitisation to channel activation. Analysis of the magnitude of Ca^{2+} release following caffeine application to permeabilised cells 'clamped' at $[Ca^{2+}]_c$ measured in non-permeabilised cells (see above; the range of experimentally determined resting $[Ca^{2+}]_c$ in intact cells is indicated by the vertical dashed lines in Fig. 1E) predicted that $N^{2386}I$ and $R^{176}Q/T^{2504}M$ exhibited hyper-activated Ca^{2+} release (216% and 228%, respectively), when compared with WT RyR2 (186%). In contrast, $L^{433}P$ was predicted to exhibit equivalent Ca^{2+} release (173%) in response to caffeine addition. These values predicted from data obtained in permeabilised cells corroborate our original assignment of $N^{2386}I$ and $R^{176}Q/T^{2504}M$, but not $L^{433}P$, as 'gain-of-function' channelopathies.

Since the amplitude and duration of Ca^{2+} release through RyR is critically dependent on the status of the ER Ca^{2+} store, we next sought to determine whether the different Ca^{2+} sensitivities of the SCD-linked mutations were due, in part, to accompanying changes in ER Ca^{2+} storage. Furthermore, it was important to ensure that the loss of Ca^{2+}-dependent inhibition was not due to depletion of the ER store following cellular permeabilisation/$[Ca^{2+}]$ manipulation. Fig. 2A shows comparable ER filling status in all cells at a range of imposed $[Ca^{2+}]_c$, indicating that our permeabilisation protocol did not significantly perturb the ER Ca^{2+} loading status. The relatively constant ER loads in these cells suggest specific SLO permeabilisation of the plasma membrane (PM), and not of intracellular organelles. Accordingly, the ER and cytoplasm remain physically and functionally compartmentalised in these cells, suggesting that alterations in channel sensitivity to $[Ca^{2+}]_c$, independent of the ER lumenal Ca^{2+} store, underpin the functional defects in SCD-linked RyR2 mutations.

Although prolonged exposure to the extremes of $[Ca^{2+}]_c$ used in our experiments would eventually impact on the ER Ca^{2+} status, our data demonstrate that our $[Ca^{2+}]_c$ manipulation protocols (i.e., <10 min equilibration) maintained ER Ca^{2+} status. However, despite there being no statistically significant differences in the ER load in these $[Ca^{2+}]_c$ 'clamped' cells, two trends are apparent. First, there is a trend toward hyper-filling the ER as $[Ca^{2+}]_c$ is increased in untransfected HEK cells, and those expressing WT and $L^{433}P$ RyR2 (where the relative gradients of the trendlines are $+0.607 \pm 0.06$ (HEK), $+0.505 \pm 0.18$ (WT RyR2), and $+0.782 \pm 0.04$ ($L^{433}P$)). HEK cells are endogenously RyR deficient, and thus the relative hyper-filling observed in our experiments may not be due to RyR-dependent processes. In contrast, $N^{2386}I$ and $R^{176}Q/T^{2504}M$ expression was associated with ER depletion as $[Ca^{2+}]_c$ increases (where relative gradients are -0.727 ± 0.20 and -0.122 ± 0.14, respectively) (Fig. 2A). These data may be indicative of a small Ca^{2+} leak from the ER through $N^{2386}I$ and $R^{176}Q/T^{2504}M$ as $[Ca^{2+}]_c$ increases, in concert with our finding that these mutations exhibit desensitised $[Ca^{2+}]_c$ inhibition (Figs. 1B and D). However, as indicated above, the ER Ca^{2+} stores in these permeabilised cells are not significantly different, and none of the SCD-linked mutations presently studied condition the cell to elevated $[Ca^{2+}]_c$ under non-stimulated conditions, indicating that in resting cells, the presence of SCD-linked mutations is functionally benign. Thus, characterisation of these mutants fully supports a model of RyR2 Ca^{2+} release channel dysfunction triggered by cellular stimulation, in keeping with the normal phenotype of SCD-susceptible individuals at rest.

The expression of WT and mutant RyR2 did not perturb the endogenous levels of SERCA, CRT, and CSQ, proteins intimately involved in intracellular Ca^{2+} handling (Figs. 2B and C). Thus, the comparable ER Ca^{2+} stores determined in Fig. 2A were underpinned by similar expression profiles of ER Ca^{2+} handling proteins, a finding that further supports our data indicating that post-activation RyR2 channel dysfunction of $L^{433}P$, $N^{2386}I$, and $R^{176}Q/T^{2504}M$ arises from altered sensitivities to $[Ca^{2+}]_c$.

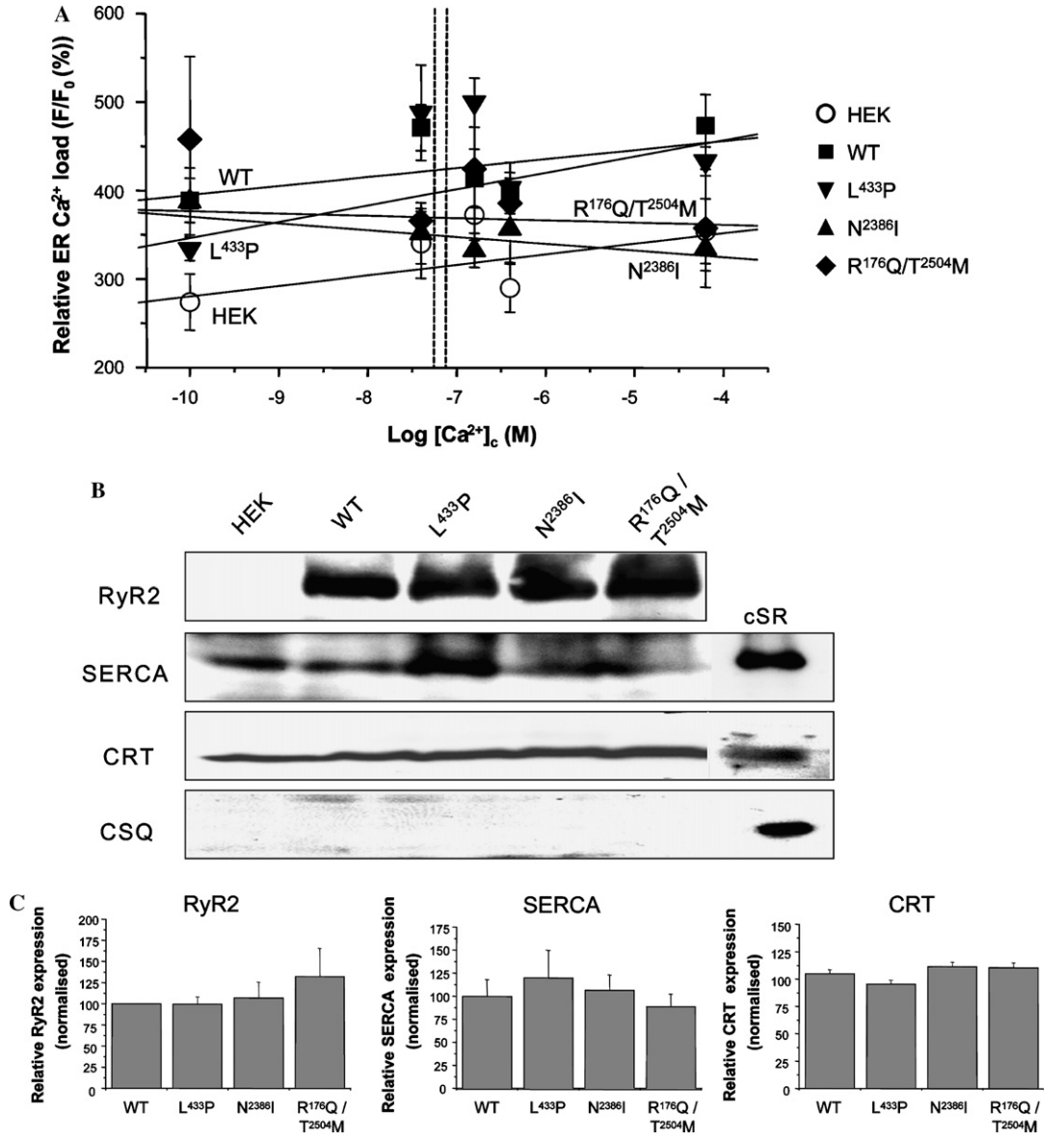

Fig. 2. The ER Ca^{2+} store status remains unaffected following manipulation of $[Ca^{2+}]_c$. (A) The ER Ca^{2+} store content was estimated in SLO-permeabilised cells at fixed $[Ca^{2+}]_c$ by determining TG (5 μM) stimulated Ca^{2+} release. Data represent the peak change in fluo-3 fluorescence following TG addition (F) relative to the resting fluorescent signals (F_o) expressed as a percentage and are given as means ± SE ($n = 3$ experiments, >15 cells analysed per experiment). The vertical dashed lines indicate the estimated ER Ca^{2+} load in non-permeabilised cells, where $[Ca^{2+}]_c$ is in the range of log$[Ca^{2+}]$ −7.1 to −7.3, were predicted to be equivalent, and in complete agreement with the experimental determination of ER Ca^{2+} loads in intact cells expressing these RyR2 mutations [16]. (B) The endogenous levels of SERCA, CRT, and CSQ were determined following immunoblot analysis of post-nuclear supernatants (SERCA and CRT, 100 μg; CSQ, 250 μg) obtained from untransfected cells (HEK) or from cells expressing WT or mutant RyR2. Rabbit cardiac homogenates (50 μg) were used as controls. (C) Densitometric analysis of recombinant RyR2 expression was normalised to the expression levels of recombinant WT RyR2 following $CaPO_4$ transfection, and SERCA and CRT levels in cells expressing recombinant WT or mutant RyR2 were normalised to the levels of SERCA and CRT determined in untransfected HEK cells. Data are plotted as means ± SE and are derived from analysis of at least four separate blots.

Discussion

We used SLO permeabilised HEK cells expressing equivalently high levels of WT and RyR2 mutations to perform the first determination of the Ca^{2+} dependence of channel activation and inhibition in living cells. SCD-linked mutations $L^{433}P$, $N^{2386}I$, and $R^{176}Q/T^{2504}M$ exhibited significantly altered Ca^{2+} sensitivity characterised by a marked loss of Ca^{2+}-dependent channel inhibition. The inhibition profiles of these mutations differed markedly from those of the WT channel which exhibited a biphasic Ca^{2+} release profile with the channel being inhibited at low (<10 nM) and high (>10 μM) $[Ca^{2+}]_c$. Our results show that in a cell-based assay, the activation and inhibition of WT RyR2 is tightly regulated by $[Ca^{2+}]_c$, in contrast to reports that WT RyR2

displays pronounced insensitivity to Ca^{2+}-dependent inhibition in vitro [28]. However, discrepant results regarding RyR2 regulation can arise from the use of particular assays to determine channel functionality ([^{3}H]ryanodine binding versus single channel reconstitution used to determine the channel's 'open' status) [27–29]. To this end, our values of Ca^{2+}-dependent activation obtained in permeabilised cells expressing WT RyR2 are in close agreement with the in vitro characterisation of the Ca^{2+}-dependence of caffeine-stimulated Ca^{2+} release through WT RyR2 in planar lipid bilayers ($EC_{50} = 42$ nM versus $EC_{50} = 93$ nM) obtained by Li and Chen [27]. Our data also confirm sensitised Ca^{2+}-dependent channel activation in the presence of caffeine (EC_{50} of 42 nM versus 260 nM in the absence of caffeine [27]), entirely consistent with the finding that caffeine sensitises the WT channel to activation by cytoplasmic [Ca^{2+}] [30]. It is also notable that as well as sensitising RyR2 to Ca^{2+} activation, caffeine also appears to dramatically sensitise RyR2 to Ca^{2+}-dependent inhibition by a factor of ~600-fold (IC_{50} 3.3 μM (this study) versus 2.1 mM in the absence of caffeine [27]). Thus, we have shown that in living cells, activation of RyR2 Ca^{2+} release is exquisitely modulated by $[Ca^{2+}]_c$ between ~50 nM and ~2 μM, entirely within the physiological Ca^{2+} range.

Despite attractive candidate mechanisms for Ca^{2+} release abnormalities through mutant RyR2, the precise mechanistic basis of RyR2 dysfunction in arrhythmia remains poorly defined [11–15]. Recently, functional characterisation of three C-terminal CPVT-linked mutations revealed that lumenal Ca^{2+} sensitisation underpinned RyR2 dysfunction, rather than RyR2 dysfunction induced by changes in ER Ca^{2+} storage per se [13]. These findings are in accord with the hypothesis of Choi et al. [4], who proposed that arrhythmic phenotype may not be absolutely dependent on altered ER Ca^{2+} store status. Consequently, it is entirely feasible that in some instances, arrhythmogenic susceptibility may arise from a distinct regulatory mechanism, i.e., mutant RyR2 exhibits an altered response to $[Ca^{2+}]_c$. We have addressed this issue and have shown that some ARVD2-linked mutations result in a loss of Ca^{2+}-dependent inactivation over a wide range of $[Ca^{2+}]_c$. After analysing the mode of Ca^{2+} release through these SCD-linked mutations, we previously proposed that channel activation would be predicted to result in prolonged $[Ca^{2+}]_c$ elevation [16]. The present study confirms and extends this concept by providing strong evidence that in these same mutations, the failure to completely terminate Ca^{2+} release following channel stimulation may arise as a consequence of a loss of Ca^{2+}-dependent inactivation (~8- to 10-fold) that would prolong the $[Ca^{2+}]_c$ elevation, and directly increase the likelihood of DAD and arrhythmia in SCD-susceptible individuals. Although, we currently do not know the precise mechanisms underlying the abnormal RyR2 Ca^{2+} release termination observed in our experiments (e.g., defective channel inactivation vs. adaptation) [31], a detailed investigation of the pathophysiological role of Ca^{2+}-dependent inactivation in terminating ER/SR Ca^{2+} release provides scope for important future study.

The molecular determinants of RyR Ca^{2+}-dependent inactivation have been proposed to lie in the C-terminus [28,29], at loci entirely separate from the mutational location of the N- and central domain locations studied presently. Nevertheless, when considering the complex conformational architecture of structural domains within the folded RyR tetramer, our data strongly support a role for the N-terminal and central domains in Ca^{2+}-dependent inactivation, and may point to SCD-linked mutational 'hot-spots' occurring at sites of key intra-RyR conformational interaction. Thus, our data are fully consistent with the determinants of RyR Ca^{2+} sensitivity being dependent on the proper, folded conformation of the intact channel. We therefore propose that the mutational loci may be important in determining the precise mechanistic basis of RyR2 dysfunction in SCD, in keeping with the suggestion that similar pathological phenotypes may arise from markedly distinct mechanistic bases [32].

Our study provides evidence for SCD-linked RyR2 mutations altering $[Ca^{2+}]_c$ sensitivity, that when taken together with reports of altered sensitivity to ER lumenal [Ca^{2+}] [14], and the finding that increased FKBP12.6 dissociation from RyR2 mutants introduces channel abnormalities [15], strongly indicates that mutation-linked RyR2 dysfunction is most unlikely to arise from a single 'unifying' mechanism. Consequently, a comprehensive analysis of all SCD-linked mutations must be performed on an individual basis in order to assign a precise mechanistic basis of Ca^{2+} release dysfunction. So far, only 31% of reported mutations have been functionally characterised, but the increasing numbers of published mutations should facilitate the correlation between the severity of disease, the mode of channel dysfunction, and the precise role of mutational loci.

The mutations characterised in this study exhibit grossly abnormal $[Ca^{2+}]_c$ sensitivity in living cells, yet we do not currently know the pathological mechanisms that manipulate $[Ca^{2+}]_c$ in such a way so as to induce the profound Ca^{2+} release dysfunction associated with the onset of VT. This is important since the large $[Ca^{2+}]_c$ changes that underpin every cardiac cycle do not, under normal circumstances, trigger arrhythmia/VT in SCD-susceptible individuals. The phenotypically normal resting state together with the stress/exercise requirement for the development of arrhythmia suggests that, in addition to mutation, gross RyR2 dysfunction requires an additional 'trigger.' Thus, a systematic evaluation of stress/exercise-induced 'factors' that exacerbate RyR2 dysfunction, e.g., other components of the cellular

Ca^{2+} handling machinery that contribute to RyR2 channel abnormalities in stressful circumstances, represents an important avenue of investigation.

Although this is the first characterisation of WT and mutant RyR2 Ca^{2+} sensitivity in a cellular context, we acknowledge some potential limitations of the study. We used caffeine to trigger RyR-dependent Ca^{2+} release, yet caffeine is not an endogenously occurring activating ligand of RyR2. However, caffeine is extensively used by a multitude of laboratories to investigate RyR channel function in biochemical and cell-based assays. The interaction between caffeine and RyR2 may be mediated by a large N-terminal domain (amino acids 305–2150) that encompasses the $L^{433}P$ locus [33]. However, $L^{433}P$ exhibits Ca^{2+}-dependent caffeine activation, and the loss of Ca^{2+}-dependent inhibition following caffeine addition is also observed with other mutations occurring at distinct loci within the polypeptide. Although they represent a non-myocytic background, we specifically used HEK cells so that our data can be interpreted in the broader context of findings from other laboratories also obtained using heterologous expression of RyR2 in HEK cells [12–15].

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