Functional heterogeneity of ryanodine receptor mutations associated with sudden cardiac death

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Abstract

Objectives: Point mutations in the cardiac ryanodine receptor (RyR2) mediate abnormal intracellular Ca\textsuperscript{2+} release and are associated with stress-induced ventricular tachycardia (VT), leading to sudden cardiac death (SCD). Although the precise molecular basis of RyR2 dysfunction in SCD remains controversial, there is consensus that the mutations characterised to date all exhibit gain-of-function Ca\textsuperscript{2+} release properties following cell stimulation. We investigated the functional impact of a distinct set of SCD-linked RyR2 mutations (L\textsuperscript{433}P, N\textsuperscript{2386}I, R\textsuperscript{176}Q/T\textsuperscript{2504}M) on intracellular Ca\textsuperscript{2+} handling. Methods: We expressed full-length recombinant human wild-type (WT) and SCD-linked RyR2 mutations in human embryonic kidney (HEK) cells, and profiled the spatial and amplitude characteristics of caffeine-evoked Ca\textsuperscript{2+} release through homo-tetrameric channels in living cells using rapid confocal laser scanning microscopy. Results: Analysis of the precise mode of Ca\textsuperscript{2+} release in HEK cells expressing RyR2 mutants demonstrated profound differences when compared with WT channels. The SCD-linked RyR2 mutations characterised in this study exhibited heterogeneous Ca\textsuperscript{2+} release profiles, including the novel observation that one of the mutants, (L\textsuperscript{433}P), exhibited a marked reduction in sensitivity to channel activation. However, all SCD-linked RyR2 mutations characterised in this study resulted in an increased duration of elevated cytoplasmic Ca\textsuperscript{2+} levels following channel activation. Conclusions: Our live cell-based data demonstrates functional heterogeneity of Ca\textsuperscript{2+} release through SCD-linked RyR2 mutants, suggesting that the mechanistic basis of RyR2 dysfunction in SCD may be more complex than previously anticipated. These findings may have profound consequences for the therapeutic modulation of RyR2 in stress-induced VT and SCD.

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1. Introduction

Ryanodine receptors (RyRs) are massive tetrameric Ca\textsuperscript{2+} release channels that underpin excitation–contraction coupling in cardiac muscle [1]. In healthy hearts, the cardiac RyR isoform (RyR2) is exquisitely modulated by a plethora of accessory proteins and cellular effectors resulting in normal Ca\textsuperscript{2+} homeostasis [2]. However, RyR2 dysregulation leads to profound perturbations in intracellular Ca\textsuperscript{2+} and is associated with heart failure and sudden cardiac death (SCD) [3–7]. To date, 24 point mutations in the human RyR2 have been linked with the autosomal dominant variant of stress-induced ventricular tachycardia (VT), a cardiac pathology characterised by delayed after-depolarisations (DADs) and cytoplasmic Ca\textsuperscript{2+} overload following physical or emotional stress, leading to SCD. The presence of RyR2 mutations identifies a subset of SCD-susceptible individuals with an earlier age of onset (8 ± 2 years compared with 20 ± 12 years non-genotyped stress-induced VT) and pronounced male bias (relative risk of 4.2 of developing syncope when compared with female subjects) [8].

Currently, the precise mechanistic basis of RyR2 dysregulation in the pathogenesis of SCD remains to be fully resolved, but may involve 12.6 kDa FK506-binding protein (FKBP12.6)-dependent [7,9] or -independent [10] mecha-
nisms, or enhanced resting activity of the RyR2 channel [11]. However, there is a general consensus that the four RyR2 mutations so far characterised (S2246L, R2474S, N3104K and R4497C) exhibit gain-of-function Ca\(^{2+}\) release following channel activation [9–11]. In the present study, we characterised the Ca\(^{2+}\) release profiles of a distinct set of RyR2 mutations recently identified in a single cohort of patients [6] and demonstrate that these mutations can be considered bona fide channelopathies. Importantly, there was marked heterogeneity in the Ca\(^{2+}\) release profiles of these mutants, which challenges the perception that all RyR2 mutations currently linked with SCD result in augmented Ca\(^{2+}\) channel functionality.

2. Materials and methods

2.1. Construction and expression of recombinant SCD-linked RyR2 mutations

The full-length cDNA sequence encoding recombinant human RyR2 tagged at the N-terminus with enhanced green fluorescent protein (eGFP) [10] was modified using oligonucleotide directed mutagenesis (Quickchange; Stratagene, Netherlands) to incorporate SCD-linked mutations [6]. Oligonucleotides were reverse-phase purified (Sigma-Genosys, Cambridge, UK) and their sequences are as follows:

- **L\(^{433}\)P:** GATTATAAGGGCCCT GATGCTCTCAG-CAAG/ L\(^{433}\)PR: CTTGCTGAGACGATCAGGCCCTTATAAC;
- **N\(^{2386}\)I:**
- **N\(^{2386}\)IF:** CTATCCACATGGGATC GCGATGACCT/GCCCT/ N\(^{2386}\)IR: AAGGTCACTGATGGGACCACATCCCATGGA-
- **TAG:**
- **R\(^{176}\)Q/T\(^{2504}\)M:** (a ‘double’ mutant, where both mutations exist on the same allele [6]):
  - **R\(^{176}\)Q:** GAAGGAGAAAAGTACAAGTTGGAGAT-
  - **GACCT/ R\(^{176}\)QR:** AGGTCACTCACCAGTTGAGAT-
  - **GACCT/T\(^{2504}\)MF:** CTGCTTCTTATGATGGCGAGCTTGCT-
  - **T\(^{2504}\)MR:** AGCACTCAAAGCTGCAATCTAAGACG;

where the underline represents the mutated codon. Mutagenesis of R\(^{176}\)Q and L\(^{433}\)P was performed using a SpeI/SanDI RyR2 fragment (−15 to 5542 bp) sub-cloned into pSL1180 (Amersham Biosciences, UK). Mutations N\(^{2386}\)I and T\(^{2504}\)M were introduced into a SanDI/KpnI RyR2 fragment (5542 to 7678 bp) in pSL1180. Full-length RyR2 containing SCD-linked mutations were created following the re-insertion of mutagenised cassettes using the restriction enzymes above. All constructs were verified by automated sequencing (ABI 3700, Applied Biosystems). Plasmid cDNAs encoding full-length wild-type (WT) and mutant GFP-tagged RyR2 were propagated in XL-10Gold *Escherichia coli* (Stratagene) following stringent procedures [12] and large-scale plasmid purification was performed using gel-based purification systems (Qiagen) to avoid degradation of the fragile RyR2 plasmid. High purity (A\(260/A_{280}>1.9\)) plasmid cDNA was transfected (4–6 μg) into human embryonic kidney (HEK) cells (1 × 10\(^{4}\) at ~70% confluency) using a calcium phosphate precipitation method [13]. Cells were maintained in Dulbecco’s Modified Eagle Medium (Invitrogen, Paisley, UK) containing foetal calf serum (10% [vol/vol]), glutamine (2 mM) and penicillin/streptomycin (100 μg/ml).

Expression of recombinant protein was verified 24–36 h post-transfection by immunoblotting analysis of cellular extracts and in situ immunofluorescent detection using an anti-GFP monoclonal antibody (clone B-2; Santa Cruz Biotechnology, CA, US) or anti-RyR2 polyclonal antiserum (pAb 1093; immunogenic epitope: human RyR2 amino acid residues 4455–4474) as previously described [10,14]. Densitometric analysis of developed blots was carried out using a densitometric scanner (GS700, Bio-Rad) and Quantity One software (Bio-Rad).

2.2. Intracellular Ca\(^{2+}\) imaging

Intracellular Ca\(^{2+}\) mobilisation in fluo3-AM (10 μM in 20% (w/v) pluroneric acid F-127 (Biotium, CA, US)) loaded cells maintained in Krebs–Ringer HEPES buffer (KRH; 120 mM NaCl, 25 mM HEPES, 4.8 mM KCl, 1.4 mM CaCl\(_2\), 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgCl\(_2\); pH 7.4) was determined following caffeine addition (0.1–40 mM, applied as a bolus in KRH) to cells on poly-l-lysine-coated glass coverslips using an RS2 confocal microscope (Leica Microsystems, Heidelberg, Germany) as described [10,14]. Cells on separate coverslips were used per application of caffeine to negate the effect of sequential caffeine application on intracellular Ca\(^{2+}\) handling in the same population of cells, i.e., all experiments were performed against a background of comparable ER Ca\(^{2+}\) load status. ER Ca\(^{2+}\) load was estimated from peak Ca\(^{2+}\) release following the addition of thapsigargin (TG, 5 μM) to cells [12]. HEK cells expressing recombinant RyR exhibit a functionally compartmentalised ER Ca\(^{2+}\) store comprising a significant caffeine-insensitive component [15], and thus we used TG, and not caffeine, to estimate total ER Ca\(^{2+}\) load in HEK cells expressing WT and mutant RyR2. In the TG experiments, RyR2 expressing cells were first identified by the addition of 0.5 mM caffeine, followed by several washes and complete...
extracellular solution exchange with fresh KRH buffer, prior to TG application. Data were acquired from regions of interest representing global Ca$^{2+}$ environments (typically approximately 50 μm$^2$), and analysed using Leica Confocal and GraphPad Prism software. Statistical analysis was performed using unpaired Student’s $t$ test.

3. Results and discussion

3.1. Expression of SCD-linked RyR2 mutations does not perturb resting cell phenotype

We achieved efficient recombinant RyR2 plasmid transfer into HEK cells (Fig. 1A), which resulted in equivalent, high level expression of full-length WT and SCD-linked RyR2 mutations (where expression relative to RyR2WT (100%) was; L433P, 99.7 ± 8.5%; N2386I, 106.9 ± 18.5%; R176Q/T2504M, 131.9 ± 33.7%; R176Q, 96.1 ± 4.3% and T2504M, 121.79 ± 16.9%) (Fig. 1B). HEK cells are widely used in the structure/function characterisation of RyR2 [9,11,16,17] since they do not express endogenous RyR (Fig. 1B), and thus represent a suitable system with which to study the functional impact of homo-tetrameric recombinant RyR2 mutants on intracellular Ca$^{2+}$ homeostasis. Direct visualisation of recombinant protein using eGFP fluorescence (Fig. 1C, top panel), or following immunodetection using a high-titre anti-RyR2 antibody (pAb1093) (Fig. 1C, middle panel) confirmed their correct targeting to the endoplasmic reticulum (ER) according to the characteristic lattice-like morphology. The near total co-incidence between endogenous eGFP fluorescence and immunolocalisation via anti-RyR2 antibody labeling of recombinant protein (Fig. 1C, lower panel (merge)) (WT RyR2, 98.7 ± 0.4%; L433P, 98.1 ± 0.7%; N2386I, 98.9 ± 0.5%; R176Q/T2504M, 84.8 ± 3.9%; R176Q, 95.2 ± 2.3%, T2504M, 95.7 ± 2.7%) corroborated our immunoblotting analysis and strongly indicated the in situ expression of full-length recombinant hRyR2. The size and morphology of cells expressing RyR2 mutants were indistinguishable from those expressing WT

![Fig. 1. Expression and intracellular localisation of WT and SCD-linked RyR2 mutations. (A) The endogenous fluorescence of eGFP-tagged hRyR2 (right panel) was used to determine the efficiency of plasmid transfection by comparison with the total number of cells seen in the phase image (left panel). Transfection efficiencies of 15–20% were routinely achieved. Bar represents 25 μm. (B) Post-nuclear supernatants (500 μg) obtained from HEK cells transiently expressing WT and mutant RyR2 were immunoblotted using a mouse monoclonal anti-GFP antibody (Santa Cruz Biotechnology) as described in the Materials and Methods. Densitometric analysis of immunoblots was performed on three separate experiments. (C) The intracellular localisation of recombinant WT and mutant RyR2 was determined using endogenous eGFP fluorescence (top panels) or via immunofluorescent detection of recombinant protein using anti-RyR2 antisera (pAb1093) (middle panels). The direct overlay of eGFP- and immunofluorescence images obtained at high resolution was used to determine the extent of co-localization of eGFP and antibody signals as previously described 10 (lower panels; merge). Bar represents 10 μm.](https://academic.oup.com/cardiovascres/article-abstract/64/1/52/298138)
RyR2, entirely consistent with previous studies showing that the resting cell phenotype is not perturbed following expression of SCD-linked RyR2 mutations [9,10].

To accurately determine changes in intracellular [Ca\(^{2+}\)] in our experiments, we used the Ca\(^{2+}\) indicator fluo3 due to its large dynamic range, low compartmentalization tendency and appropriate apparent Ca\(^{2+}\) binding affinity (we determined an apparent \(K_d\) [\(K_{app}\)] of 733 ± 143 nM) [18]. Although these are desirable properties and are ideally suited for the present study, fluo3 has a nearly identical excitation/emission profile to eGFP and thus it is not possible to easily distinguish between their respective fluo- rescences. Nevertheless, the contribution of eGFP fluorescence to our Ca\(^{2+}\) measurements was negligible for two reasons. Firstly, unlike fluo3, eGFP fluorescence is entirely Ca\(^{2+}\)-independent and remained unaltered following caffeine-induced Ca\(^{2+}\) mobilization from intracellular stores (Fig. 2A). Consequently, our determination of Ca\(^{2+}\) release in this study, which was calculated following the measurement of relative changes in intracellular fluorescence, is entirely attributable to Ca\(^{2+}\) dependent changes in fluo3 signals. Secondly, the typical fluorescence of ER localised recombinant eGFP-tagged RyR2 protein was negligible when compared with total cellular fluorescence following intracellular loading with fluo3-AM (Fig. 2B). Taken together, these findings provide good evidence that fluo3 can be used to accurately determine intracellular Ca\(^{2+}\) mobilization in our experimental system.

3.2. SCD-linked RyR2 mutation L\(^{433}\)P exhibits desensitised caffeine-induced activation

Dysfunctional Ca\(^{2+}\) release through SCD-linked RyR2 mutations is manifested following cellular stimulation [9,10] and thus we characterised the temporal and amplitude properties of caffeine-evoked Ca\(^{2+}\) transients in single, living wild-type (untransfected) HEK cells, or those expressing WT or mutant RyR2 (Fig. 3A). We used caffeine to trigger RyR2 Ca\(^{2+}\) release in these experiments since caffeine sensitises RyR Ca\(^{2+}\) activation. Untransfected HEK cells did not exhibit caffeine-induced Ca\(^{2+}\) release, confirming the lack of functional RyR in these cells (Fig. 3A). Consistent with previous reports of heterologous expression of recombinant RyR2 in null-cell systems [12,14,16,19], we did not detect Ca\(^{2+}\) sparks in our experiments. However, dose–response relationships constructed from caffeine-activated Ca\(^{2+}\) release in HEK cells expressing recombinant WT, or mutant, RyR2 revealed significant functional heterogeneity between the individual RyR2 mutants and when compared with WT RyR2 (Fig. 3B). RyR2 mutants N\(^{2386}\)I and R\(^{1767}\)/T\(^{2504}\)M exhibited enhanced sensitivity to caffeine activation (Fig. 3B), and augmented peak Ca\(^{2+}\) release (Figs. 3C and 4A), in complete accord with a current model of RyR2 mutant hyper-sensitisation leading to augmented cytoplasmic Ca\(^{2+}\) levels which underpin the pathogenesis of SCD (Fig. 3A and B). In stark contrast, Ca\(^{2+}\) release through the L\(^{433}\)P mutation was characterised by a significantly right-shifted dose response to caffeine (i.e., markedly desensitised activation) (Fig. 3B), and peak Ca\(^{2+}\) release that was indistinguishable to that determined through WT RyR2, but was significantly decreased when compared with other SCD-linked RyR2 mutations (Fig. 4A). These findings represent the first characterisation of an SCD-linked RyR2 mutant that exhibits markedly reduced sensitivity to channel activation, presumably resulting in depressed RyR2 activity in situ. Consequently, this characterisation of a desensitised RyR2 mutation fundamentally challenges the current perception that all SCD-linked RyR2 mutations represent ‘gain-
of-function’ channelopathies. In this context, it should be noted that in cardiomyocytes, RyR2 inhibition dramatically perturbs intracellular Ca\(^{2+}\) fluxes resulting in subcellular alternans [20], and thus depression of RyR2 L\(^{433}\)P channel function may represent an alternative mechanism in the pathogenesis of SCD. Although this is the first report of a
desensitised RyR2 mutation, channelopathies arising from ‘loss-of-function’ mutations in Na⁺ (SCN5A) or K⁺ (HERG and KCNE1) channels are associated with cardiac pathology and sudden death [21–24].

3.3. Analysis of the amplitude and temporal characteristics of Ca²⁺ release through RyR2 mutations

We also investigated the temporal profile of Ca²⁺ handling in cells expressing WT and mutant RyR2. There was no significant difference observed between the time to peak Ca²⁺ release following activation of WT and mutant RyR2 (Fig. 4B), but this parameter is dependent on both the amplitude of Ca²⁺ release following caffeine addition (Fig. 4A) and the rate of Ca²⁺ release, which exhibited significant heterogeneity between mutant and WT RyR2 (Fig. 4C). We also measured significant differences in the Ca²⁺ transient decay properties exhibited between individual RyR2 mutants and when compared with WT RyR2 (Fig. 4D). The time taken for caffeine-induced Ca²⁺ transients to decay to half peak amplitude represents the rate of Ca²⁺ removal from the cytosol via sequestration into intracellular organelles (particularly the ER) or its extrusion from the cell through plasma membrane (PM) localized Ca²⁺ pumps and exchangers [1]. However, our caffeine application protocol (i.e., no wash-out step, thereby leading to a persistent exposure to caffeine) would result in a sustained increase in open probability of RyR2 that would markedly diminish the ER Ca²⁺ re-uptake capacity in these cells [1,25]. However, HEK cells are endogenously RyR deficient and therefore we cannot exclude the possibility that ER micro-domains lacking recombinant RyR2 exist in our transfected cells. Thus, although we do not currently know the precise contribution of ER mediated sequestration in restoring cytoplasmic [Ca²⁺] to resting levels in these experiments, it is likely to represent a minor component, and therefore the Ca²⁺ transient decay following RyR2 activation is almost entirely due to PM-mediated Ca²⁺ extrusion or uptake into mitochondria.

Despite the marked amplitude and temporal heterogeneity that exists between SCD-linked RyR2 mutations and when compared with WT RyR2, analysis of the precise mode of Ca²⁺ release through SCD-linked RyR2 mutants (peak Ca²⁺ release versus time to peak Ca²⁺ release versus rate of Ca²⁺ release versus rate of Ca²⁺ sequestration/extrusion) predicted that the net effect of these RyR2 mutations would result in significantly augmented cytoplasmic [Ca²⁺] following channel activation, a characteristic of VT 8. This can be clearly appreciated for mutants N2386I and R176Q/T2504M where both mutants display hyper-sensitive caffeine-induced Ca²⁺ release (Fig. 3B), and significantly augmented peak cytoplasmic Ca²⁺ levels when compared with WT RyR2 following cellular activation (Fig. 3B and 4A). Importantly though, the augmented cytoplasmic Ca²⁺ levels following cellular activation also holds true for cells expressing the L433P mutation. Although
the RyR2 L433P mutation is less sensitive to activation, once it has been fully activated there is a similar peak Ca\(^{2+}\) release to that determined in cells expressing WT RyR2 (Fig. 4A). However, the more rapid rise in cytoplasmic Ca\(^{2+}\) and the significantly prolonged Ca\(^{2+}\) transient (Fig. 4A and D) would result in a sustained elevation in cytoplasmic Ca\(^{2+}\) for considerably longer durations than would occur following activation of cells expressing WT RyR2. Thus, it is important to stress that despite significant functional heterogeneity, the finding that caffeine activation of all RyR2 mutations characterised in this study resulted in augmented cytoplasmic Ca\(^{2+}\) levels following cellular stimulation, is in compliance with the occurrence of cytoplasmic Ca\(^{2+}\) overload underlying VT. In this context, our data draws close parallels with the observed functional heterogeneity of skeletal muscle RyR (RyR1) mutations associated with malignant hyperthermia (MH), where profound functional differences between RyR1 mutants underpin similar disease phenotypes [26]. Further emphasising the similarity of pathogenic RyR1 and RyR2 mutations, the RyR2 R163C mutation characterised in this study is the cardiac equivalent of a hyper-sensitive MH-linked RyR1 mutation (R16C) [27]. In our experiments, we evoked Ca\(^{2+}\) release through recombinant RyR2 using caffeine that activates the channels via increased sensitivity to ambient [Ca\(^{2+}\)]. In vivo RyRs are modulated by localised Ca\(^{2+}\) environments [2], although caffeine activation of RyR2 Ca\(^{2+}\) release may not necessarily reflect the physiological activation of RyR2 channels in native cardiac muscle.

The amplitude and temporal characteristics of caffeine-induced Ca\(^{2+}\) release determined above may be influenced by other cellular factors e.g. the relative activity of ER and plasma membrane Ca\(^{2+}\) pumps, cytoplasmic and ER Ca\(^{2+}\) buffers [1], the co-ordinated actions of which help shape cytoplasmic Ca\(^{2+}\) transients. Furthermore, the amplitude of Ca\(^{2+}\) release evoked by caffeine is critically dependent on the filling status of the ER Ca\(^{2+}\) store. Importantly, we determined comparable ER Ca\(^{2+}\) stores in cells expressing SCD-linked mutations N238I (0.97 ± 0.12), L433P (0.80 ± 0.08), R176Q/T2504M (1.22 ± 0.07), R176Q (1.13 ± 0.12) and T2504M (0.86 ± 0.10), when compared to the ER Ca\(^{2+}\) load determined in cells expressing WT RyR2 (1.00 ± 0.12). These data (mean ± S.E.M., n>25 cells in each instance) did not achieve statistical significance even at values of \(p \leq 0.05\). Thus, although we cannot completely rule out that the expression of SCD-linked RyR2 mutations results in a more generalised dysfunction in cellular Ca\(^{2+}\) handling rather than identifying dysfunction of mutant RyR2 per se, the determination of similar ER Ca\(^{2+}\) loads strongly suggests that our data highlights bona fide differences in the Ca\(^{2+}\) release properties of these SCD-linked RyR2 channels. However, further work is necessary to more precisely define the functional impact of RyR2 mutations on other aspects of cellular Ca\(^{2+}\) handling.

The use of RyR-deficient cells, notably the extensive use of HEK cells, has permitted the functional characterisation of recombinant RyR2 channels [9,11,12,14,16–18,28] separated from the additional complexities associated with RyR2 expression in cardiomyocytes, where the co-incorporation of WT endogenous RyR2 subunits into recombinant tetramers must also be considered. Using these expression systems, valuable information regarding the functional characteristics of recombinant RyR2 has been generated from the study of purified homo-tetrameric channels, isolated from their cellular environment. However, the insights into recombinant RyR2 function following heterologous expression in RyR-deficient cells should be interpreted in the knowledge that in its native environment RyR2 is regulated via localised signalling events within a macromolecular complex comprising numerous accessory proteins [28,34]. Thus, an important consideration is that recombinant RyR2 channels produced in null-cell systems may not exist in a macromolecular organisation, since many of these accessory proteins are absent. Despite this, the functional properties of RyR2 derived from null-cell models closely resemble those observed with native RyR2 channels from cardiac tissue [16,19,29,30]. The relative contribution of specific macromolecular complex components to RyR2 channel modulation remains to be elucidated. However, we propose that the functional similarities between recombinant and native RyR2 channels may be explained by two possibilities; either (i) the RyR2 macromolecular complex that exists in native tissue is sufficiently robust to withstand extraction from cardiomyocytes, yet, the co-purified proteins do not regulate the RyR channel outside its native cellular environment, or (ii) the accessory proteins in the RyR macromolecular complex are dissociated during RyR purification. These issues remain to be conclusively resolved, but it is clear that the present experimental approach is advantageous since it provides a platform to functionally characterise recombinant RyR2 in a living cell-based context and thereby supports the classification of these homo-tetrameric SCD-linked RyR2 mutations as bona fide channelopathies.

It has been hypothesised that defective intra-RyR2 interaction may underpin the pathogenesis of VT [31], and interestingly, the mutation loci in R176Q/T2504M (mutations that co-segregate with the affected phenotype) map to two regions of the RyR2 polypeptide proposed to mediate autoregulation of channel activity [32]. We investigated whether the presence of these two mutations in the same RyR2 polypeptide potentiated their respective impact on RyR2 Ca\(^{2+}\) release functionality. Figs. 3 and 4 show that recombinant RyR2 channels containing either single mutations R176Q or T2504M exhibited increased peak Ca\(^{2+}\) release when compared with WT RyR2, but demonstrated significantly different Ca\(^{2+}\) release profiles when compared with the ‘double’ R176Q/T2504M mutant and with WT RyR2. This data indicates that although these mutations represent channelopathies in their own right, the characteristics of the R176Q / T2504M ‘double’ mutation cannot simply be extrapolated from the functional effects of the single mutations, pointing to an additional level of complexity in the
mechanistic basis of RyR2 dysregulation in SCD pathogenesis which requires further experimentation.

Recently, Wehrens et al. [7] have established a link between electrical instability and RyR2 dysfunction in cardiac arrhythmia using a transgenic mouse model of RyR2 dysregulation, and stabilisation of the closed state of RyR2 appears to be an attractive and feasible therapy in the management of heart failure and arrhythmia [33]. However, our data provides the first evidence that there may not be a unifying mechanism underpinning RyR2 dysfunction in stress-induced VT and SCD, and thus the molecular basis of aberrant Ca\(^{2+}\) release in SCD appears to be more complex than had previously been anticipated. Consequently, our demonstration of functional heterogeneity in SCD-linked RyR2 mutations may have profound consequences for therapeutic modulation of RyR2 in the pathogenesis of VT and SCD. Furthermore, our data clearly points to the requirement for a comprehensive functional characterisation of newly identified RyR2 mutations on an individual basis.

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