Ryanodine Receptor Mutations Associated With Stress-Induced Ventricular Tachycardia Mediate Increased Calcium Release in Stimulated Cardiomyocytes

Christopher H. George, Gemma V. Higgs, F. Anthony Lai

Abstract—Ca\(^{2+}\) release from the sarcoplasmic reticulum mediated by the cardiac ryanodine receptor (RyR2) is a fundamental event in cardiac muscle contraction. RyR2 mutations suggested to cause defective Ca\(^{2+}\) channel function have recently been identified in catecholaminergic polymorphic ventricular tachycardia (CPVT) and arrhythmogenic right ventricular dysplasia (ARVD) affected individuals. We report expression of three CPVT-linked human RyR2 (hRyR2) mutations (S\(^{2246}\)L, N\(^{4100}\)K, and R\(^{4497}\)C) in HL-1 cardiomyocytes displaying correct targeting to the endoplasmic reticulum. N\(^{4100}\)K also localized to the Golgi apparatus. Phenotypic characteristics including intracellular Ca\(^{2+}\) handling, proliferation, viability, RyR2:FKBP12.6 interaction, and beat rate in resting HL-1 cells expressing mutant hRyR2 were indistinguishable from wild-type (WT) hRyR2. However, Ca\(^{2+}\) release was augmented in cells expressing mutant hRyR2 after RyR activation (caffeine and 4-chloro-m-cresol) or \(\beta\)-adrenergic stimulation (isoproterenol). RyR2:FKBP12.6 interaction remained intact after caffeine or 4-CMC activation, but was dramatically disrupted by isoproterenol or forskolin, an activator of adenylate cyclase. Isoproterenol and forskolin elevated cyclic-AMP to similar magnitudes in all cells and were associated with equivalent hyperphosphorylation of mutant and WT hRyR2. CPVT-linked mutations in hRyR2 did not alter resting cardiomyocyte phenotype but mediated augmented Ca\(^{2+}\) release on RyR-agonist or \(\beta\)-AR stimulation. Furthermore, equivalent interaction between mutant and WT hRyR2 and FKBP12.6 was demonstrated. (Circ Res. 2003;93:531-540.)

Key Words: ryanodine receptors • mutations • ventricular tachycardia • stress • cardiomyocytes

Normal cardiac E-C coupling requires the precise coordination of ionic currents and molecular defects in ion channels ("channelopathies") are causative of cardiac pathophysiology including long-QT syndrome, ventricular fibrillation (VF), and ventricular tachycardia (VT).\(^{1-3}\) Ca\(^{2+}\) is the most important ion involved in the cardiac muscle contraction and is rapidly mobilized from intracellular stores into the sarcoplasm to trigger the activation of contractile elements. Termination of these events requires the removal of sarcoplasmic Ca\(^{2+}\) via sequestration into the sarcoplasmic/endoplasmic reticulum (SR/ER) through Ca\(^{2+}\)-ATPase (SERCA) activity and extrusion of Ca\(^{2+}\) via the Na\(^+\)–Ca\(^{2+}\) exchanger (NCX).\(^{1}\) Duration and amplitude of Ca\(^{2+}\) efflux from the SR is regulated by cardiac ryanodine receptors (RyR2), large homotetrameric complexes that are organized into functional Ca\(^{2+}\) release units via interaction with a multitude of accessory proteins.\(^{2}\) Recently, defective regulation of RyR2, which leads to abnormal cellular Ca\(^{2+}\) handling, has been implicated in heart failure, hypertrophy, and arrhythmia.\(^{5-8}\) To date, 21 point mutations in hRyR2 have been reported in individuals affected by catecholaminergic polymorphic VT (CPVT) and arrhythmogenic right ventricular dysplasia (ARVD), and defective RyR Ca\(^{2+}\) channel function is implicated in the disease phenotype.\(^{9-12}\) Interestingly, these hRyR2 mutations cluster in highly conserved regions corresponding to mutation-containing domains of the skeletal muscle isoform (RyR1) associated with malignant hyperthermia (MH).\(^{13}\) This positional clustering of mutation loci together with the requirement for a "trigger" in both CPVT (physical/emotional stress) and MH (inhalational anesthetic) raises the possibility that CPVT/ARVD is a cardiac equivalent of MH, and indeed, certain MH-linked RyR1 mutations (eg, R\(^{614}\)C) are associated with a stress-induced phenotype. Chronic administration of \(\beta\)-blockers or implantable cardiac defibrillators are currently used to control CPVT although these do not represent optimal therapeutic strategies.\(^{8}\) Thus, understanding the molecular events underlying the disease phenotype is critical for the development of novel therapeutic approaches.

We determined the functional impact of CPVT-linked mutations in the human recombinant RyR2 (hRyR2) on intracellular Ca\(^{2+}\) handling in HL-1 cardiomyocytes. RyR2 mutations (S\(^{2246}\)L, N\(^{4100}\)K, and R\(^{4497}\)C) were expressed in HL-1 cells and we demonstrate that the resting phenotype (resting cytoplasmic [Ca\(^{2+}\)] ([Ca\(^{2+}\)]), ER Ca\(^{2+}\) load, beat rate, cell

Original received April 10, 2003; revision received August 4, 2003; accepted August 6, 2003.
From the Wales Heart Research Institute, Department of Cardiology, University of Wales College of Medicine, Cardiff, UK.
Correspondence to Dr Christopher H. George, Wales Heart Research Institute, University of Wales College of Medicine, Heath Park, Cardiff, UK CF14 4XN. E-mail georgech@cf.ac.uk.
© 2003 American Heart Association, Inc.
Circulation Research is available at http://www.circresaha.org
DOI: 10.1161/01.RES.0000091335.07574.86

531
size, and proliferation) were indistinguishable from cells expressing nonmutant WT hRyR2. The interaction between RyR2 and FKBP12.6 was intact in resting cells expressing WT or mutant hRyR2. However, activation of Ca\(^{2+}\) release using mechanistically different RyR agonists [caffeine and 4-chloro-m-cresol (4-CMC)] or β-adrenergic (β-AR) stimulation (isoproterenol) was associated with significantly augmented Ca\(^{2+}\) release through mutant hRyR2. The RyR2:FKBP12.6 complex remained intact after caffeine or 4-CMC addition but was severely disrupted after β-AR stimulation, which was associated with equivalent hyperphosphorylation of WT and mutant hRyR2. Importantly, we determined equivalent dissociation of FKBP12.6 from both WT and mutant hRyR2 after β-AR stimulation. Our data provide direct evidence that mutations in hRyR2 associated with CPVT mediates augmented Ca\(^{2+}\) release in stimulated HL-1 cardiomyocytes, which may underlie the disease phenotype.

Materials and Methods

Construction of eGFP-Tagged WT and Mutant hRyR2

A fragment of pC GFP-C3 (Clontech) (35 to 1330 bp) was amplified by PCR, digested with MluI/SpeI, and inserted into MluI/SpeI digested pcDNA3/hRyR2.\(^{14}\) This strategy placed eGFP at the N-terminus of hRyR2 separated by a four amino acid spacer (Thr-Ser-Gly-Ser). To generate the S\(^{2246}\)L point mutation, an EcoRV cassette from hRyR2 (4015 to 7013 bp) was transferred into pSL1180 (Amersham) and the TCG (Ser) codon was replaced with T7G (Leu) using oligonucleotide directed mutagenesis (Quickchange; Stratagene). A similar strategy was used to generate N\(^{3104}\)K [AAC (Asn) to AAAG (Lys)] and R\(^{4497}\)C [CGC (Arg) to TGCC (Cys)] using an NhEl/XhoI (10015 to 15335 bp) fragment of hRyR2. Full-length hRyR2 mutants were generated by the ligation of mutated cassettes into pcDNA3/hRyR2 using EcoRV (S\(^{2246}\)L) or FseI/XhoI (N\(^{3104}\)K, R\(^{4497}\)C) strategies. All constructs were verified by automated sequencing (ABI 3700, Applied Biosystems).

Analysis of RyR2 and FKBP Expression in HL-1 Cardiomyocytes

HL-1 cardiomyocytes were cultured on a gelatin (0.02% [wt/vol])/fibronectin (10 μg/mL) matrix and were maintained in Claycomb media (JRH Biosciences) supplemented with fetal calf serum (10% [vol/vol]), penicillin (100 U/mL), and streptomycin (100 μg/mL).\(^{15}\) Cells were transiently transfected with plasmid DNA using Lipofectamine2000 (Invitrogen) as described previously.\(^{14}\) The expression levels of endogenous and recombinant RyR2 were determined after immunoblot analysis of cellular fractions using anti-RyR2 (pAb129), and recombinant GFP-tagged hRyR2 was detected using anti-GFP (Clontech) as described.\(^{14}\) Anti-phosphoserine (clone 16B4, Calbiochem) was used to determine the phosphorylation status of RyR2. FKBP12 and 12.6 were detected using rabbit polyclonal antiserum, which cross-reacts with both FKBP12 and 12.6 isoforms.\(^{16}\) Densitometric analysis was performed using a GS700 scanner and Quantity One software (Biorad) as described in the expanded Materials and Methods section in the online data supplement available at http://www.circresaha.org. For direct analysis of FKBP12.6:hRyR2 interaction, communoprecipitation assays were performed on HL-1 cell lysates using pAb129 and anti-FKBP to immunoprecipitate and immunoblot, respectively (see online data supplement).

Immunofluorescent localization of endogenous RyR2 and FKBP was performed using antibodies pAb129 and N-19 (Santa-Cruz Biotechnology), respectively, followed by detection using donkey anti-rabbit Alexa 488 and donkey anti-goat Alexa 546 conjugated antibodies (Molecular Probes), respectively.\(^{14}\) Recombinant GFP-tagged WT and mutant hRyR2 were directly localized using eGFP fluorescence (Ex 488 nm, Em 511 nm) and in these cells, FKBP was detected using an Alexa 647-linked secondary antibody as above.

Phenotypic Characterization of HL-1 Cardiomyocytes

The Ca\(^{2+}\)-dependent fluorescence of calcium orange (10 μmol/L) in HL-1 cardiomyocytes was determined using a confocal microscope (SP2, Leica).\(^{16}\) ER Ca\(^{2+}\) load was estimated from peak Ca\(^{2+}\) release after the addition of thapsigargin (5 μmol/L) to cells.\(^{14}\) Caffeine (0.01 to 40 mmol/L), 4-CMC (0.01 μmol/L to 10 mmol/L), and isoproterenol (0.1 mmol/L to 10 μmol/L) were used to mobilize intracellular Ca\(^{2+}\) in cells maintained in KRH buffer containing 1.3 mmol/L CaCl\(_2\).\(^{14}\) RyR2 and β-AR were inhibited by the addition of ryanodine (1 mmol/L) and nadolol (100 μmol/L), respectively. Data were acquired in bidirectional scan mode (130 ms/frame) at 512×512 pixel resolution.

Cell surface area and beating rate (see online data supplement) were measured using confocal microscopy (Leica). Cell viability and proliferation were determined as described previously.\(^{16}\) In phenotypic analysis of transfected cells, data were collected from “GFP-positive” cells.

Measurement of Intracellular [cAMP]

Intracellular [cAMP] was measured using an enzyme-linked immunoassay (ELA) (Amersham) after stimulation of cells with caffeine (10 mmol/L), 4-CMC (1 mmol/L), isoproterenol (1 μmol/L), or forskolin (5 μmol/L). Reactions were terminated by the addition of dodecyltrimethylammonium bromide (2.5% [wt/vol]) and intracellular [cAMP] was calculated using a calibration curve constructed from known [cAMP] (12.5 to 3200 fmol).

Results

Immunoblot analysis of microsomal fractions demonstrates that transfected HL-1 cardiomyocytes express significantly higher levels of full-length RyR2 (∼560 kDa) than untransfected cells (Figure 1Aa), and that expression of recombinant WT and mutant hRyR2 is essentially equivalent (hRyR2 242±24%; S\(^{2246}\)L 221±29%; N\(^{3104}\)K 259±36%; R\(^{4497}\)C 270±37%; HL-1 100%). RyR2 is the only endogenously expressed RyR isofrom in HL-1 cells, as RyR1 and RyR3 were undetectable using isofrom-specific antibodies (data not shown). An anti-GFP antibody enabled monitoring of recombinant WT and mutant hRyR2 expression distinct from endogenous RyR2, confirming high-level, equivalent expression of recombinant protein (Figure 1Ab). Immunoblots of total cell lysates using antisera recognizing both FKBP12 and FKBP12.6,\(^{16}\) revealed FKBP12 expression of ∼4-fold higher level than FKBP12.6, and that both their cellular levels remained unchanged after expression of recombinant hRyR2 (Figure 1Ac). However, only FKBP12.6 was associated with microsomal fractions obtained from cells expressing WT and mutant hRyR2 (Figure 1Ad) and this association was completely abolished after rapamycin treatment (5 μmol/L) (Figure 1Ac), consistent with specific protein:protein interaction occurring between FKBP12.6 and WT and mutant RyR2. The elevation of hRyR2 in transfected cells (Figure 1Aa) accompanied the parallel increase in FKBP12.6 association with microsomes, a phenomenon observed with cells expressing recombinant WT or mutant hRyR2 (Figure 1Ad). In contrast, we did not detect FKBP12 in microsomal fractions, although it was abundant in postmicrosomal supernatants (Figure 1Af). Communoprecipitation studies using pAb129 confirmed that...
A specific, comparable interaction occurred between FKBP12.6 and hRyR2 (0.88 ± 0.18), S2246L (1.06 ± 0.14), N4104K (0.91 ± 0.16), and R4497C (0.87 ± 0.12) when compared with FKBP12.6 association with endogenous RyR2 in resting HL-1 cardiomyocytes (assigned 1.0) (Figure 1Ag), which was ablated after incubation of immune complexes with rapamycin (Figure 1Ah). The coimmunoprecipitation studies demonstrate that interaction between FKBP12.6 and CPVT-linked hRyR2 mutations is indistinguishable from that determined with WT hRyR2 in resting HL-1 cardiomyocytes and agrees with data obtained using the microsomal association assays (Figures 1Ad and 1Ae).

Endogenous RyR2 or recombinant hRyR2, S2246L, N4104K, and R4497C (Figure 1B) were all correctly targeted to the ER as determined by the localization of calreticulin, an ER resident protein (see online data supplement). However, N4104K was also localized to a perinuclear concentration (Figure 1B), which was identified as the Golgi apparatus.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Size, μm²</th>
<th>Viability, %</th>
<th>Growth Rate, %</th>
<th>ER Ca²⁺ Load, %</th>
<th>Beat Rate, 1/min</th>
<th>ER phenotypic characteristics of HL-1 cardiomyocytes expressing WT and mutant hRyR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-1</td>
<td>732 ± 76</td>
<td>86 ± 11</td>
<td>100</td>
<td>100</td>
<td>152 ± 24</td>
<td>Shoots, viability, growth rate, Ca²⁺ load, and beat rate.</td>
</tr>
<tr>
<td>+ hRyR2</td>
<td>817 ± 82</td>
<td>79 ± 12</td>
<td>87 ± 7</td>
<td>136 ± 24*</td>
<td>158 ± 32</td>
<td>Significant differences in ER Ca²⁺ load and beat rate compared to WT.</td>
</tr>
<tr>
<td>+ S2246L</td>
<td>834 ± 75</td>
<td>73 ± 16</td>
<td>89 ± 14</td>
<td>156 ± 29*</td>
<td>139 ± 30</td>
<td>Significant differences in ER Ca²⁺ load and beat rate compared to WT.</td>
</tr>
<tr>
<td>+ N4104K</td>
<td>743 ± 69</td>
<td>84 ± 13</td>
<td>96 ± 8</td>
<td>134 ± 25*</td>
<td>137 ± 23</td>
<td>Significant differences in ER Ca²⁺ load and beat rate compared to WT.</td>
</tr>
<tr>
<td>+ R4497C</td>
<td>831 ± 64</td>
<td>81 ± 15</td>
<td>110 ± 12</td>
<td>142 ± 32*</td>
<td>141 ± 31</td>
<td>Significant differences in ER Ca²⁺ load and beat rate compared to WT.</td>
</tr>
</tbody>
</table>

Data were obtained from the following number of cells per experiment: size (31 ± 5), viability (92 ± 14), ER Ca²⁺ load (27 ± 5), and beat rate (21 ± 5). All data are given as mean ± SEM (n = 4).

*P < 0.01 when compared with untransfected HL-1 cells.
using an antibody to Golgi 58K protein (see online data supplement). Image analysis demonstrated a significant interaction (Merge) between membrane localized WT/mutant hRyR2 (green) and FKBP (red) (Figures 1B and 1C). However, much FKBP, presumably FKBP12 (Figures 1Ad and 1Af), remained in the cytoplasm. Taken together, these data demonstrate that in resting HL-1 cells, a specific interaction occurs between FKBP12.6 and mutants S2246L, N4104K, and R4497C that is indistinguishable from that which occurs with WT hRyR2. The validity of our confocal imaging approach to accurately determine the in situ association of the RyR2:FKBP12.6 complex was confirmed by the separation of fluorescent signals from RyR2 and FKBP after treatment of the cells with rapamycin (Figures 1B and 1C). These findings are entirely complementary with data obtained using immunoblotting of microsomal fractions or coimmunoprecipitation assays (Figure 1A).

Cell size, viability, and proliferation remained unaltered after expression of recombinant WT or mutant RyR2s in resting HL-1 cells, and we determined an equivalent increase in ER Ca\(^{2+}\) storage capacity in cells expressing WT or mutant hRyR2 (Table). However, mutant RyR2s exhibited enhanced sensitivity to caffeine activation (Figure 2A), and we measured a significant increase in the magnitude and duration of Ca\(^{2+}\) release after addition of caffeine (40 mmol/L) to cells expressing S2246L, N4104K, and R4497C when compared with untransfected cells or those expressing WT hRyR2 (Figures 2A and 2B).

**Figure 2.** Augmented intracellular Ca\(^{2+}\) release, but intact RyR2:FKBP12.6 interaction through mutant hRyR2 after caffeine stimulation. **A,** Proportional Ca\(^{2+}\) release when compared with maximal Ca\(^{2+}\) release (100%) after caffeine addition to HL-1 cells expressing WT or mutant hRyR2 was determined. Data are given as mean ± SEM (n=4). *P<0.01. **B,** Ca\(^{2+}\) release profiles after the addition of caffeine (40 mmol/L) (arrow) to HL-1 cells expressing WT or mutant hRyR2 (top traces) or cells preincubated with ryanodine (1 mmol/L) (bottom traces). Data are plotted as relative fluorescence units (F.U.). **C** and **D,** The magnitude of Ca\(^{2+}\) release relative to that measured in untransfected HL-1 cells (100%) (C) and the duration of the Ca\(^{2+}\) transient (D) after caffeine addition (40 mmol/L) were calculated. Data are plotted as mean ± SEM (n=6). **P<0.01 when compared with untransfected HL-1 cells and cells expressing mutant hRyR2 and *P<0.01 when compared with control cells and those expressing WT hRyR2.**  

**E,** Colocalization (yellow) between endogenous RyR2 (HL-1) or recombinant hRyR2, S2246L, N4104K, and R4497C (green) and FKBP (red) after caffeine addition (40 mmol/L) to HL-1 cardiomyocytes. RyR2:FKBP12.6 coincidence was determined as described in Figure 1. Bar=10 μm. **F,** Association of FKBP12.6 with microsomes (200 μg) from untreated cells (–, open bars) or from cells exposed to caffeine (40 mmol/L) (+, open bars). Densitometric values were obtained as optical density (OD; signal intensity per mm\(^2\)) and are plotted as mean ± SEM from three separate experiments.
2B through 2D). The caffeine-induced Ca\(^{2+}\) release in cells expressing either WT hRyR2 or mutant hRyR2 was increased when compared with untransfected HL-1 cells due to the augmentation of the ER Ca\(^{2+}\) store (Table).\(^{14}\) Ryanodine (1 mmol/L) completely abolished caffeine-induced Ca\(^{2+}\) release demonstrating that channels assembled from S2246L, N4104K or R4497C were sensitive to ryanodine modification (Figure 2B, lower traces). Importantly, the augmented Ca\(^{2+}\) release determined in cells expressing mutant RyR2s in response to caffeine stimulation was not underscored by dissociation of the RyR:FKBP12.6 complex, which remained intact (Figures 2E and 2F).

Mutant RyR2s also displayed an increased sensitivity to activation by 4-CMC (Figure 3A), and 4-CMC caused a sustained elevation in [Ca\(^{2+}\)], the magnitude of which was significantly augmented in cells expressing mutant hRyR2 (Figures 3B and 3C). The rate of Ca\(^{2+}\) mobilization after 4-CMC addition was similar in all cells (Figure 3D) and Ca\(^{2+}\) release could be inhibited by preincubation with ryanodine (Figure 3B, lower traces). RyR2:FKBP12.6 association remained intact after stimulation of cells with 4-CMC (Figures 3E and 3F). The Golgi localization of N4104K persisted after caffeine and 4-CMC stimulation of cells (Figs. 2E and 3E, respectively).

\(\beta\)-Adrenergic status is implicated in the pathogenesis of the CPVT phenotype,\(^8\) and we investigated the effect of \(\beta\)-AR activation of cells expressing mutant hRyR2. Mutant RyR2s exhibited a left-shifted dose response after addition of isoproterenol, a \(\beta\)-AR agonist that induces VT in individuals carrying hRyR2 mutations\(^{10}\) (Figure 4A). The maximal Ca\(^{2+}\)
release elicited by isoproterenol (1 μmol/L) was equivalently augmented in cells expressing WT or mutant RyR2s (Figure 4C), but we determined a significant increase in the duration of the Ca\(^{2+}\) transient in cells expressing S2246L, N4104K, or R4497C (Figure 4D). Ryanodine (1 mmol/L) (middle traces) or nadolol (200 μmol/L) (bottom traces), Data are plotted as relative fluorescence units (F.U.). C and D, Magnitude of Ca\(^{2+}\) release relative to that measured in untransfected HL-1 cells (100%) (C) and the duration of Ca\(^{2+}\) transients (D) after isoproterenol addition (1 μmol/L) were determined. Data are plotted as mean±SEM (n=6) and *P<0.005 when compared with untransfected HL-1 cells and **P<0.005 when compared with untransfected HL-1 cells and HL-1 cells expressing WT hRyR2. E, Colocalization between endogenous RyR2 (HL-1) or recombinant hRyR2, S2246L, N4104K, and R4497C (green) and FKBP (red) after isoproterenol addition. Bar=15 μm. F, Association of FKBP12.6 with microsomal fractions (200 μg) obtained from untreated cells (−, open bars) or cells exposed to isoproterenol (1 μmol/L) (+, filled bars). Densitometric values were obtained as optical density (IOD: signal intensity per mm\(^2\)) and are plotted as mean±SEM from three separate experiments. *P<0.01.

We used forskolin, a potent activator of adenylate cyclase, to determine the effect of direct stimulation of downstream effectors of the β-adrenergic signaling cascade on RyR2:FKBP12.6 interaction. In concert with our data from receptor-mediated activation of the β-adrenergic cascade (Figure 4), forskolin dramatically reduced RyR2:FKBP12.6 interaction but importantly, the dissociation was equivalent in localization and microsomal association assays demonstrated that isoproterenol addition disrupted RyR2:FKBP12.6 interaction in HL-1 cardiomyocytes, in agreement with the finding that β-AR stimulation causes the dissociation of the RyR2:FKBP12.6 complex, but importantly, we determined equivalent dissociation between FKBP12.6 and WT and mutant RyR2s (Figures 4E and 4F).
WT and mutant RyR2 expressing cells (Figures 5A and 5B). The dramatic disruption of the RyR:FKBP12.6 complex induced by isoproterenol and forskolin persisted after ryanodine-mediated inhibition of RyR indicating its ablation of RyR2:FKBP12.6 interaction was independent of the open/closed status of RyR (data not shown).

PKA-mediated hyperphosphorylation of RyR2 has been detected in failing hearts, although the functional consequences of this remain highly controversial. We determined whether (1) β-AR stimulation resulted in differential phosphorylation of mutant and WT hRyR2 and (2) intracellular elevations in [cAMP] after β-AR activation were equivalent in all cells. Microsomal proteins (>80 kDa) obtained from HL-1 cells expressing WT hRyR2 exhibited a broad ladder of bands following SDS-PAGE separation (Figure 6A, lane 1) and RyR2 was identified using pAb129 (Figure 6A, lane 2). An anti-phosphoserine antibody (16B4) confirmed that RyR2 was phosphorylated in nonstimulated cells (Figure 6A, lane 3). Endogenous RyR2, recombinant WT, and mutant RyR2s were phosphorylated to equivalent levels in untreated cells (endogenous RyR2, 1.0; hRyR2, 0.88±0.11; S2246L, 0.81±0.09; N4104K, 0.75±0.12; R4497C, 0.97±0.14) (Figure 6Ba), and the phosphorylation status was unaltered after stimulation of cells with caffeine or 4-CMC (Figure 6Bb and 6Bc, respectively). The increased phosphorylation signals from transfected cells (Figure 6B) proportionally reflect the increased expression levels of both WT and mutant RyR2s (Figure 1A). β-AR stimulation using isoproterenol significantly but equivalently increased the phosphorylation of WT hRyR2 (1.91±0.23) and mutants S2246L (2.28±0.31), N4104K (2.32±0.29), and R4497C (1.98±0.23) (Figure 6Bd) relative to the phosphorylation status of WT and mutant hRyR2 determined in untreated cells (Figure 6Ba and data above). Similarly, forskolin addition also resulted in hyperphosphorylation of mutant hRyR2 (S2246L, 3.11±0.31; N4104K, 3.65±0.4; R4497C, 3.20±0.35), which was indistinguishable from that of WT (2.89±0.39) (Figure 6Be). Staurosporine, a broad-spectrum inhibitor of protein kinases, completely inhibited RyR2 phosphorylation (Figure 6Bf). Thus, β-adrenergic stimulation resulted in hyperphosphorylation of hRyR2, but our data clearly demonstrate that the extent of phosphorylation of CPVT-linked hRyR2 mutants S2246L, N4104K and R4497C was indistinguishable from that of WT hRyR2 in HL-1 cardiomyocytes.

Isoproterenol and forskolin treatment was associated with significant elevations in intracellular [cAMP], which was similar in untransfected HL-1 cells or those expressing WT or mutant RyR2s. The increased phosphorylation signals from transfected cells (Figure 6B) proportionally reflect the increased expression levels of both WT and mutant RyR2s (Figure 1A). β-AR stimulation using isoproterenol significantly but equivalently increased the phosphorylation of WT hRyR2 (1.91±0.23) and mutants S2246L (2.28±0.31), N4104K (2.32±0.29), and R4497C (1.98±0.23) (Figure 6Bd) relative to the phosphorylation status of WT and mutant hRyR2 determined in untreated cells (Figure 6Ba and data above). Similarly, forskolin addition also resulted in hyperphosphorylation of mutant hRyR2 (S2246L, 3.11±0.31; N4104K, 3.65±0.4; R4497C, 3.20±0.35), which was indistinguishable from that of WT (2.89±0.39) (Figure 6Be). Staurosporine, a broad-spectrum inhibitor of protein kinases, completely inhibited RyR2 phosphorylation (Figure 6Bf). Thus, β-adrenergic stimulation resulted in hyperphosphorylation of hRyR2, but our data clearly demonstrate that the extent of phosphorylation of CPVT-linked hRyR2 mutants S2246L, N4104K and R4497C was indistinguishable from that of WT hRyR2 in HL-1 cardiomyocytes.
RyR2s (Figure 6C). However, the extent of equivalent hyperphosphorylation of WT and mutant RyR2s after stimulation of the β-AR cascade was proportional to the increased cellular levels of cAMP (forskolin/isoproterenol) (Figure 6C). The smaller caffeine-induced increase in [cAMP] did not result in hRyR2 hyperphosphorylation (Figure 6Bb) or dissociation of the RyR2:FKBP12.6 complex (Figures 2E and 2F), indicating that in our experiments, increases in cellular [cAMP] greater than 5-fold over basal levels are associated with disruption of the RyR2:FKBP12.6 complex.

**Discussion**

We expressed recombinant GFP-tagged hRyR2 channels (~560 kDa), which correctly localized to the ER and formed functional Ca^{2+} release channels in HL-1 cardiomyocytes. Resting cell phenotype was unaltered after expression of hRyR2 containing CPVT-linked mutations (S2246L, N4104K, and R4497C), and thus our data obtained in cardiomyocytes strongly suggest that there is normal and appropriate regulation of CPVT-linked mutant hRyR2 channels in nonstimulated cells. In our experiments, we did not observe the increased basal “activity” previously associated with expression of a CPVT-linked RyR2 mutation (mouse R1406C) in HEK cells. However, it is likely that appropriate regulation of RyR2 requires the precise interaction of a multitude of accessory proteins, some of which may be absent in the RyR-deficient cells used by Jiang and colleagues.
Intracellular Ca\(^{2+}\) release was significantly augmented in cells expressing mutant hRyR2 after addition of RyR agonists (caffeine and 4-CMC) or β-AR stimulation (isoproterenol), providing strong evidence that abnormal Ca\(^{2+}\) release during periods of emotional or physical stress may underlie the delayed afterdepolarizations and Ca\(^{2+}\) overload phenomena associated with CPVT.\(^8,10\) Defective regulation of RyR2 via PKA mediated hyperphosphorylation and dissociation of FKBP12.6 is implicated in heart failure.\(^5,21\) Recently, the selective dissociation of FKBP12.6 from ARVD-linked RyR2 mutants has also been suggested,\(^22\) although these data were not corroborated using mutations in full-length RyR2 sequence and intriguingly, a robust interaction was demonstrated between FKBP12.6 and RyR1, an interaction that does not occur in vivo.\(^9\) Importantly, although our data indicate that β-AR–induced RyR2 hyperphosphorylation is associated with disruption of the FKBP12.6:RyR2 complex, in situ localization studies and direct measurement of FKBP12.6:hRyR2 protein:protein interaction revealed that the extent of dissociation was equivalent in cells expressing WT and mutant hRyR2 (Figure 6). Thus, it is unlikely that differential phosphorylation of mutant hRyR2 or selective dissociation of FKBP12.6 from CPVT-linked hRyR2 mutants is causative of the disease phenotype. However, our results do not exclude the possibility that in stimulated cells, abnormal Ca\(^{2+}\) release through mutant hRyR2 is underscored by differential sensitivity to equivalent phosphorylation. Also, although our results strongly suggest that dissociation of the RyR2:FKBP12.6 complex is not causative of CPVT phenotype, it has recently been reported that genetic ablation of FKBP12.6 is associated with exercise-induced fatal arrhythmia in mice expressing wild-type RyR2.\(^23\) Hyperphosphorylation of RyR2 and RyR2:FKBP12.6 dissociation was proportional to the elevation in [cAMP]. However, elevated [cAMP] also modulates phospholamban and Na\(^+\) channels,\(^18,24\) which would directly alter intracellular Ca\(^{2+}\) homeostasis. Furthermore, although our results strongly support a causative role for aberrant regulation of Ca\(^{2+}\) release via RyR2 in CPVT, a CPVT-linked mutation in calsequestrin has been identified\(^26\) and defective β-AR signaling via RyR2-independent mechanisms is implicated in other tachycardias.\(^26\) Therefore, although we found elevated ER Ca\(^{2+}\) release on β-AR stimulation occurred specifically via mutant RyR2 (ie, was ryanodine sensitive) (Figure 4), one must consider the role of accessory cellular factors in coordinating intracellular Ca\(^{2+}\) release.

RyR agonists (caffeine and 4-CMC) significantly augmented the magnitude of Ca\(^{2+}\) release through mutant hRyR2, yet the RyR2:FKBP12.6 complex remained intact (Figures 2 and 3, respectively). This finding strongly indicates that there are defects in FKBP12.6-independent regulation of hRyR2 mutants in HL-1 cardiomyocytes that leads to increased Ca\(^{2+}\) release on cell stimulation. The precise nature of these cellular regulatory components remains to be defined. Currently, chronic administration of β-blockers are used in the management of CPVT.\(^8,10\) However, due to the many intracellular targets of the β-AR signaling cascade, our results indicate that the precise in situ regulation of mutant RyR2 channel function represents an attractive and feasible therapeutic strategy.\(^17\) The precise molecular mechanisms by which mutations in hRyR2 mediate augmented Ca\(^{2+}\) release in stimulated cells are currently unknown, and clearly a more detailed understanding of structure-function aspects of hRyR2 will be necessary to develop novel therapeutic strategies in the management of CPVT/ARVD.

Cardiac arrhythmia susceptibility genes so far identified predominantly encode ion channels\(^2,3\) and our study suggests that mutations in RyR2 are bona fide additions to the list of channelopathies. An intriguing aspect of the hRyR2 mutations is that they are presented in a cardiac specific context but it is unlikely that these mutations are imprinted in the heart. RyR2 is expressed in other tissues (eg, brain and kidney) and CPVT-linked hRyR2 mutations may in the future be linked with noncardiac pathologies as is the case with K\(_{\text{c,LQT1}}\) mutations associated with long-QT syndrome, which are also linked with epilepsy and congenital neural deafness.\(^3\) It is also likely that CPVT-linked hRyR2 mutations may be associated with other cardiac abnormalities, as is observed with SCN5A mutants that have been identified in several arrhythmogenic phenotypes including long-QT syndrome and VF.\(^3\)

Acknowledgments

This work was supported by a British Heart Foundation Research Fellowship (FS2000020) to C.H.G. and British Heart Foundation Grants (PG98169 and PG99087) to F.A.L. We thank W. Claycomb for HL-1 cardiomyocytes, J. Mackrill for anti-FKBP12/12.6 antibody, and N.L. Thomas for helpful discussions.

References


Ryanodine Receptor Mutations Associated With Stress-Induced Ventricular Tachycardia Mediate Increased Calcium Release in Stimulated Cardiomyocytes

Christopher H. George, Gemma V. Higgs and F. Anthony Lai

Circ Res. 2003;93:531-540; originally published online August 14, 2003;
doi: 10.1161/01.RES.0000091335.07574.86

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/93/6/531

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2003/09/16/93.6.531.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Supplementary information.

Materials and Methods.

HL-1 cells (passage 86), derived from the AT-1 murine atrial cardiomyocyte tumour lineage were a gift from W. Claycomb (Louisiana State University Medical Center). Claycomb media was obtained from JRH Bioscience and all other cell culture materials were from Invitrogen. Forskolin, 4-chloro-m-cresol (4-CMC), thapsigargin, staurosporine, and mouse anti-phosphoserine antibody were from Calbiochem. The peptide anti-GFP antibody and plasmid pEGFP-C3 were from Clontech. All fluorescently conjugated secondary antibodies and calcium orange were from Molecular Probes. All molecular biology reagents were from Promega except for the oligonucleotide-directed mutagenesis kit (Quikchange) which was from Stratagene. HPLC- purified oligonucleotides were obtained from Sigma-Genosys. All HRP-conjugated secondary antibodies and electrophoresis reagents were from BiOrad. XL-10 Gold (Stratagene) was the only bacterial strain used for propagation of plasmid containing full-length hRyR2 or its mutants; DH5α (Invitrogen) were used for propagation of all other plasmids. All other reagents were from Sigma unless specified.

Construction of GFP-tagged hRyR2 and CPVT-linked mutant hRyR2.

A fragment of peGFP-C3 (35-1330bp; encoding the CMV promoter and open reading frame of enhanced green fluorescent protein (eGFP, Clontech) was amplified using PCR and Pfu polymerase and the following oligonucleotides

CMV-GFP-F; 5' gcacgcgtgtccatagccatatatgcg 3'; GFP-SpeI-R; 5' gcactagctctggtaagctcgtcc 3'

MluI and SpeI sites respectively are underlined.

The complementary oligonucleotide pairs used to generate hRyR2 mutants were:

S^{2246}L: (5’ ctggatgtggctgcagctttggtgatggataataag 3’ / 5’ ttcattattatccatcaccaagctgcagccacatccag 3’).

N^{4104}K: (5’ gtcgccgtccttctgacaaagctctctgagcacatgccc 3’ / 5’ ggccatgtgctcagagagctttgtcagaaggacggcgac 3’).

R^{4497}C: (5’ ctctcaaatatttctgtgaacaattttacatcagagag 3’ / 5’ tctcatgtttgaatggtcagacgaagatcata 3’).

HL-1 cardiomyocyte culture and transfection.

A detailed description of the generation and culture conditions required to maintain the contractile phenotype of HL-1 cardiomyocytes has been published by Claycomb and colleagues. Plasmid cDNA encoding GFP-tagged hRyR2^{WT} or hRyR2 containing CPVT-linked mutations were prepared from magnetic-bead based purification strategies (Purefection, Promega) and cDNA (4µg) was complexed with 5µl Lipofectamine according to manufacturer's protocol (Invitrogen). Cells (2x10^5) were transfected in 60mm dish at ~50% confluency) with the DNA:lipid complexes in serum- and antibiotic- depleted Claycomb media for 15h at 37°C. After this period, cells were further cultured in supplemented Claycomb
media (as above) for 24h prior to experiments. For imaging experiments, cells were trypsinised and reseeded on glass coverslips at a density of approximately 100 cells/mm². Transfection efficiencies of ~30-40% were routinely achieved (see Figure 1S) and these were critically dependent on the yield and integrity of the purified hRyR2 plasmid.

HL-1 cardiomyocytes exhibit synchronous beating in confluent monolayers but do not contract when subconfluent. Subconfluent (non-beating) HL-1 cardiomyocytes were used in all experiments except in the determination of cellular beat rate where confluent (beating) cell monolayers were used.

**Figure 1S.** Transfection efficiency of HL-1 cardiomyocytes with hRyR2.

HL-1 cardiomyocytes were transfected with GFP-hRyR2WT (see above for details) and 36h post-transfection cells were visualised using phase microscopy (left panel) or using GFP fluorescence (right panel) (Axioplan 200, Zeiss). Bar represents 20µm.

**Characterisation of RyR2 and FKBP expression in HL-1 cardiomyocytes.**

iii) **Immunoblot analysis.**

Cells were harvested 48h post-transfection and cell lysates, microsomal fractions and post-microsomal supernatants were obtained as described. For analysis of RyR expression / phosphorylation levels, cell fractions were separated using 4% (v/v) acrylamide SDS-PAGE and for resolution of FKBP12 and 12.6 isoforms, 20% (v/v) acrylamide was used. Following electrophoresis, proteins were transferred to PVDF membranes and processed for immunoblotting as described previously except for using mouse monoclonal anti-phosphoserine antibody (clone 16B4; Calbiochem) where membranes were blocked with TBS-T containing BSA (3% (w/v)). Following incubation with primary antibodies (pAb129 (rabbit), 1:500; anti-GFP peptide antibody (rabbit), 1: 1000; anti-FKBP12 / 12.6 (rabbit), 1:1000; anti-phosphoserine (16B4) (mouse); 0.1µg/ml). Membranes were incubated with secondary antibodies conjugated to horse-radish peroxidase (HRP) and following incubation with chemiluminescent substrates (Supersignal; Pierce) signals were recorded using a CCD camera (Biorad).
ii) Co-immunoprecipitation.

HL-1 cardiomyocytes or those transiently expressing WT or mutant hRyR2 (1 x 10^7) were lysed in IP buffer (150 mmol/L NaCl; 20 mmol/L Tris; 2 mmol/L DTT; 0.3% (w/v) CHAPS; pH7.4) on ice and were incubated for 2h at 4°C with constant rotation. Following centrifugation (1,500 xg, 10mins) to remove cell debris, supernatants (500µg) were incubated with protein-G agarose (10µl; Santa-Cruz Biotechnology) for 2h at 4°C. Following this pre-clearing and removal of the protein-G agarose (15000 x g, 1min), pAb129 (1:250 dilution) was added to the supernatant and incubation was continued for 36h at 4°C with constant rotation. In some experiments, rapamycin (5 µmol/L) was added to disrupt RyR2:FKBP12.6 interaction and for negative controls, pre-immune serum was added during this incubation. Following incubation in the presence or absence of pAb129 and rapamycin, 10µl protein-G agarose was added to the samples and incubation continued for 4h at 4°C. Immune complexes were pelleted by centrifugation (15000xg, 1min) and pellets were washed three times in cold IP buffer and a final wash in 50 mmol/L Tris (pH6.8). Pellets were resuspended in SDS-sample buffer and processed for SDS-PAGE (20% (v/v) acrylamide) and immunoblotting as previously described 2.

iii) Immunofluorescent localisation.

Immunofluorescent localisation of endogenous and recombinant protein was performed as described 2,3. Endogenous RyR2 was localised using pAb129 and localisation of FKBP12 and 12.6 was performed using pAbN-19 ((goat), 1:500) (Santa-Cruz Biotechnology) followed by detection using donkey-anti rabbit Alexa488 and donkey anti-goat Alexa546 antibodies, respectively (Molecular Probes). Recombinant GFP-tagged hRyR2 was localised using eGFP fluorescence which was indistinguishable to signals obtained following immunolocalisation of GFP-hRyR2 using pAb129 (data not shown). ER and Golgi compartments were localised using rabbit anti-calreticulin (1:1000) (Santa-Cruz Biotechnology) and mouse anti-Golgi 58K protein (1:250) (Sigma) which were visualised using donkey anti-rabbit Alexa488- conjugated antibodies and goat anti-mouse Alexa546 antibodies, respectively (see Figure 3S).

Assessment of beating rate of HL-1 cardiomyocytes.

Confluent cultures of HL-1 cardiomyocytes which had established beating were imaged every 50ms (20Hz, > video rate) using a confocal microscope with acquisition set at 128 x 64 pixel resolution (Leica SP2). Images were collected for 60-90s, assembled into a continuous format (Leica Confocal Software) and the beating rate was determined from analysis of these movie files. For analysis of transfected cells, data acquisition was restricted to 'GFP-positive' cells.

Determining the phosphorylation status of RyR2.

Chemiluminescent signals from cellular RyR2 (pAb129) and from phosphophorylated RyR2 (16B4) were quantified using QuantityOne software (Biorad). The signal densities obtained
from densitometric analysis of RyR2 and phospho-RyR2 immunoblots in cells expressing WT or mutant hRyR2 were normalised to RyR and phospho-RyR2 signals obtained from untransfected HL-1 cells, respectively (which were assigned 1), following subtraction of background densities (see Figure 6). The ratio of phosphoRyR2 : protein RyR2 were calculated in each instance from these measurements. Data was derived from the analysis of three separate experiments in each instance.

In order to estimate the relative phosphorylation status of WT and mutant hRyR2 following cellular treatments when compared with untreated cells, the ratio of phospho-RyR2:RyR2 protein was calculated following densitometric analysis as described above, and this ratio was directly compared with the phosphorylation status of the respective recombinant hRyR2 determined in untreated cells. For example, for WT hRyR2 the ratio of phospho-RyR2:RyR2 protein in untreated cells = 0.88 when normalised to endogenous RyR2 signals (assigned 1) (Fig. 6A (a)). In isoproterenol treated cells, the ratio of phosphoRyR2:RyR2 protein for WT hRyR2 = 1.65 (Fig. 6A (d)). Thus the relative isoproterenol induced hyperphosphorylation of WT hRyR2 = 1.65/0.88 = 1.87.

**Measurement of intracellular [cAMP].**

The intracellular generation of cAMP was stopped by the addition of dodecyl-trimethylammonium bromide (2.5% (w/v)) 90-120s after the addition of caffeine, 4-CMC or isoproterenol. This represented the most feasible time to terminate cAMP generation following maximal agonist-induced Ca$^{2+}$ mobilisation (see Figures 2B, 3B and 4B). [cAMP] was measured using a competitive enzyme-linked assay (EIA) (Amersham) using the non-acetylation protocol and [cAMP] was calculated following calibration of the system using known concentrations of cAMP (12.5-3200fmol).
Results.

![Image of ER and Golgi visualization in HL-1 cardiomyocytes]

*Figure 3S. ER and Golgi visualisation in HL-1 cardiomyocytes.*

The ER (green) and Golgi apparatus (red) in HL-1 cardiomyocytes were immunolocalised as described above. Bar represents 15µm.

References.