The CPVT-associated RyR2 mutation G230C enhances store overload-induced Ca\(^{2+}\) release and destabilizes the N-terminal domains

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INTRODUCTION

CPVT (catecholaminergic polymorphic ventricular tachycardia) is an inherited life-threatening arrhythmogenic disorder. CPVT is caused by DADs (delayed after-depolarizations) that are induced by spontaneous Ca\(^{2+}\) release during SR (sarcoplasmic reticulum) Ca\(^{2+}\) overload, a process also known as SOICR (store-overload-induced Ca\(^{2+}\) release). A number of mutations in the cardiac ryanodine receptor RyR2 are linked to CPVT. Many of these CPVT-associated RyR2 mutations enhance the propensity for SOICR and DADs by sensitizing RyR2 to luminal or luminal/cytosolic Ca\(^{2+}\) activation. Recently, a novel CPVT RyR2 mutation, G230C, was found to increase the cytosolic, but not the luminal, Ca\(^{2+}\) sensitivity of single RyR2 channels in lipid bilayers. This observation led to the suggestion of a SOICR-independent disease mechanism for the G230C mutation. However, the cellular impact of this mutation on SOICR is yet to be determined. To this end, we generated stable inducible HEK (human embryonic kidney)-293 cell lines expressing the RyR2 WT (wild-type) and the G230C mutant. Using single-cell Ca\(^{2+}\) imaging, we found that the G230C mutation markedly enhanced the propensity for SOICR and reduced the SOICR threshold. Furthermore, the G230C mutation increased the sensitivity of single RyR2 channels to both luminal and cytosolic Ca\(^{2+}\) activation and the Ca\(^{2+}\)-dependent activation of [\(^{3}\)H]ryanodine binding. In addition, the G230C mutation decreased the thermal stability of the N-terminal region (amino acids 1–547) of RyR2. These data suggest that the G230C mutation enhances the propensity for SOICR by sensitizing the channel to luminal and cytosolic Ca\(^{2+}\) activation, and that G230C has an intrinsic structural impact on the N-terminal domains of RyR2.

Key words: intracellular calcium release, ryanodine receptor (RyR), sarcoplasmic reticulum (SR), SR Ca\(^{2+}\) overload, ventricular tachycardia.
SOICR-induced DADs and triggered activities [3,6,7,16,23,24, 27–29]. A fundamental unresolved question is how CPVT-associated RyR2 mutations enhance the propensity for SOICR. Given that SOICR occurs during SR Ca\(^{2+}\) overload, the response of RyR2 to elevated SR store/luminal Ca\(^{2+}\) is likely to be a critical determinant of SOICR. To test this hypothesis, single-channel recordings in lipid bilayers have been employed to directly determine the impact of a number of CPVT RyR2 mutations on the sensitivity of single RyR2 channels to luminal Ca\(^{2+}\) activation in the near absence of cytosolic Ca\(^{2+}\). These single-channel analyses revealed that all of the CPVT RyR2 mutations tested preferentially increase the sensitivity of the channel to luminal Ca\(^{2+}\) activation [23,24]. Some of the mutations sensitize the channel to both luminal and cytosolic Ca\(^{2+}\) activation [23,30]. Thus increased sensitivity to SR luminal Ca\(^{2+}\) activation or to both luminal and cytosolic Ca\(^{2+}\) activation may explain their enhanced propensity for spontaneous Ca\(^{2+}\) waves during SR Ca\(^{2+}\) overload. However, it is also important to emphasize that mutation-induced sensitization of RyR2 to luminal and/or cytosolic Ca\(^{2+}\) activation alone does not produce spontaneous Ca\(^{2+}\) waves in the steady state. SR Ca\(^{2+}\) overload is required to elevate the SR Ca\(^{2+}\) content to a threshold level to trigger spontaneous Ca\(^{2+}\) waves [31].

Recently, Marks and co-workers reported a novel CPVT RyR2 mutation, G230C, that only increases the sensitivity of RyR2 to cytosolic, but not luminal, Ca\(^{2+}\) activation [32]. On the basis of this observation, it was proposed that the SOICR mechanism is not required to explain CPVT associated with G230C. Instead, the diastolic SR Ca\(^{2+}\) ‘leak’ caused by a leftward shift in the cytosolic Ca\(^{2+}\) response of RyR2 was proposed to be a common mechanism underlying CPVT. However, there was no information on the direct impact of the G230C mutation on SOICR in the study by Meli et al. [32]. Furthermore, given the requirement of spontaneous Ca\(^{2+}\) waves for producing triggered activities and the requirement of threshold SR Ca\(^{2+}\) content for inducing spontaneous Ca\(^{2+}\) waves, it is unclear how the small SR Ca\(^{2+}\) leak alone without the involvement of SOICR could be arrhythmogenic.

To determine directly the impact of the G230C mutation on SOICR, we generated stable inducible HEK (human embryonic kidney)-293 cell lines that express the RyR2 WT (wild-type) and the G230C mutant, and assessed their SOICR properties. We found that the G230C mutation increased the propensity for SOICR by reducing the ER (endoplasmic reticulum) luminal Ca\(^{2+}\) threshold at which SOICR occurs. We further demonstrated that the G230C mutation increased the sensitivity of single RyR2 channels to both cytosolic and luminal Ca\(^{2+}\) activation. Thermal stability analysis of the purified N-terminal region (amino acids 1–547) of the RyR2 WT and G230C mutant proteins suggests that the G230C mutation has an intrinsic structural effect on the N-terminal domains of RyR2. These data together with those reported previously support the view that enhanced SOICR is a common feature of RyR2 mutations associated with CPVT.

**MATERIALS AND METHODS**

**Construction of the RyR2-G230C mutation**

The point mutation G230C in mouse RyR2 was performed by the overlap extension method using PCR [33,34]. Briefly, an NheI/AflIII fragment with mutation G230C was obtained by overlapping PCR. The NheI/AflIII fragment was removed from the PCR product and was used to replace the corresponding WT fragment in the full-length RyR2 cDNA in the expression plasmid pcDNA5. The mutation and sequence of the PCR product were confirmed by DNA sequencing.

**Generation of stable inducible HEK-293 cell lines**

Stable inducible HEK-293 cell lines expressing RyR2 WT or the G230C mutant were generated using the Flp-In T-REx Core Kit from Invitrogen. Briefly, Flp-In T-REx-293 cells were co-transfected with the inducible expression vector pcDNA5/FRT/TO containing the mutant cDNAs and the pOG44 vector encoding the Flp recombinase in 1:5 ratios using the calcium phosphate precipitation method. The transfected cells were washed with PBS 24 h after transfection followed by a change into fresh medium for 24 h. The cells were then washed again with PBS, harvested and plated on to new dishes. After the cells had attached (~4 h), the growth medium was replaced with a selection medium containing 200 μg/ml hygromycin (Invitrogen). The selection medium was changed every 3–4 days until the desired number of cells was grown. The hygromycin-resistant cells were pooled, aliquoted (1 ml) and stored at −80°C. These positive cells are believed to be isogenic, because the integration of RyR2 cDNA is mediated by the Flp recombinase at a single FRT site.

**Western blotting**

HEK-293 cell lines grown for 24 h after induction were washed with PBS (137 mM NaCl, 8 mM Na\(_2\)HPO\(_4\), 1.5 mM KH\(_2\)PO\(_4\), and 2.7 mM KCl, pH 7.4) plus 2.5 mM EDTA and harvested in the same solution by centrifugation for 8 min at 700 g in an IEC Centra-CL2 centrifuge. The cells were then washed with PBS without EDTA and centrifuged again at 700 g for 8 min. The PBS-washed cells were solubilized in a lysis buffer containing 25 mM Tris, 50 mM Hepes (pH 7.4), 137 mM NaCl, 1% CHAPS, 0.5% soya bean phosphatidylcholine, 2.5 mM DTT and a protease inhibitor mix (1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μg/ml aprotinin and 0.5 mM PMSF). This mixture was incubated on ice for 1 h. Cell lysate was obtained by centrifuging twice at 16000 g in a microcentrifuge at 4°C for 30 min to remove unsolubilized materials. The RyR2 WT and G230C mutant proteins were subjected to SDS/PAGE (6% gel) [35] and transferred on to nitrocellulose membranes at 45 V for 18–20 h at 4°C in the presence of 0.01% SDS [36]. The nitrocellulose membranes containing the transfected proteins were blocked for 30 min with PBS containing 0.5% Tween 20 and 5% (w/v) non-fat skimmed milk powder. The blocked membrane was incubated with the anti-RyR antibody (34c) (1:1000 dilution) and then incubated with the secondary anti-[mouse IgG (heavy and light)] antibodies conjugated to horseradish peroxidase (1:20000 dilution). After washing for 5 min three times, the bound antibodies were detected using an enhanced chemiluminescence kit from Pierce.

**Single-cell cytosolic Ca\(^{2+}\) imaging of HEK-293 cells**

Cytosolic Ca\(^{2+}\) levels in stable inducible HEK-293 cells expressing RyR2 WT or G230C channels were monitored using single-cell Ca\(^{2+}\) imaging and the fluorescent Ca\(^{2+}\) indicator dye fura 2/AM (fura 2 acetoxymethyl ester) as described previously [23,24]. Briefly, cells grown on glass coverslips for 18–22 h after induction by 1 μg/ml tetracycline were loaded with 5 μM fura 2/AM in KRH (Krebs–Ringer–Hepes) buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 6 mM glucose, 1.2 mM MgCl\(_2\), and 25 mM Hepes, pH 7.4) plus 0.02% pluronic F-127 and 0.1 mg/ml BSA for 20 min at room temperature (23°C). The coverslips were then mounted in a perfusion chamber (Warner Instruments) on an inverted microscope (Nikon TE2000-S). The cells were perfused continuously with KRH buffer containing increasing extracellular...
Ca\(^{2+}\) concentrations (0, 0.1, 0.2, 0.3, 0.5, 1.0 and 2.0 mM). Caffeine (10 mM) was applied at the end of each experiment to confirm the expression of active RyR2 channels. Time-lapse images (0.25 frame/s) were captured and analysed with Complus Simple PCI 6 software. Fluorescence intensities were measured from regions of interest centred on individual cells. Only cells that responded to caffeine were analysed. The filters used for fura 2 imaging were \( \lambda_{ex} = 340 \pm 26 \) nm and \( \lambda_{em} = 387 \pm 11 \) nm, and \( \lambda_{em} = 510 \pm 84 \) nm with a dichroic mirror (410 nM).

**Single-cell luminal Ca\(^{2+}\) imaging of HEK-293 cells**

Luminal Ca\(^{2+}\) levels in HEK-293 cells expressing RyR2 WT or RyR2 G230C were measured using single-cell Ca\(^{2+}\) imaging and the FRET (fluorescence resonance energy transfer)-based ER luminal Ca\(^{2+}\)-sensitive cameleon protein D1ER as described previously [28]. The cells were grown to 95% confluence in a 75 cm\(^2\) flask, passaged with PBS and plated in 100-mm-diameter tissue culture dishes at ~10% confluence 18–20 h before transfection with D1ER cDNA using the calcium phosphate precipitation method. After transfection for 24 h, the growth medium was then changed to an induction medium containing 1 \( \mu \)g/ml tetracaine (Sigma). After induction for ~22 h, the cells were perfused continuously with KRH buffer containing various concentrations of CaCl\(_2\) (0, 1 and 2 mM) and tetracaine (1 mM) for estimating the store capacity or caffeine (20 mM) for estimating the minimum store level by depleting the ER Ca\(^{2+}\) stores at room temperature (23°C). Images were captured with Complus Simple PCI 6 software every 2 s using an inverted microscope (Nikon TE2000-S) equipped with an S-Fluor 20×/0.75 objective. The filters used for D1ER imaging were \( \lambda_{ex} = 436 \pm 20 \) nm for CFP and \( \lambda_{em} = 500 \pm 20 \) nm for YFP, and \( \lambda_{ex} = 465 \pm 30 \) nm for CFP and \( \lambda_{em} = 535 \pm 30 \) nm for YFP with a dichroic mirror (500 nm). The amount of FRET was determined from the ratio of the light emission at 535 and 465 nm.

\( ^{3}H \)Ryanodine binding

The cells were grown to 95% confluence in a 75 cm\(^2\) flask, passaged with PBS and plated in 100-mm-diameter tissue culture dishes at ~10% confluence 18–20 h before transfection with RyR2 WT or G230C mutant cDNAs. After transfection for 24 h, the cells were harvested and lysed in lysis buffer containing 25 mM Tris, 50 mM Hepes, pH 7.4, 137 mM NaCl, 1% CHAPS, 0.5% egg phosphatidylcholine and 2.5 mM DTT and a protease inhibitor mix (1 mM benzamidine, 2 \( \mu \)g/ml leupeptin, 2 \( \mu \)g/ml pepstatin A, 2 \( \mu \)g/ml aprotinin and 0.5 mM PMSF) on ice for 60 min. The cell lysate was obtained after removing the unsolubilized materials by centrifugation twice in a microcentrifuge at 4°C for 30 min each. Equilibrium \( ^{3}H \)ryanodine binding to cell lysates was performed as described previously [37] with some modifications. \( ^{3}H \)Ryanodine binding was carried out in a total volume of 300 \( \mu \)l binding solution containing 30 \( \mu \)l of cell lysate, 100 mM KCl, 25 mM Tris, 50 mM Hepes (pH 7.4), 5 nM \( ^{3}H \)ryanodine and CaCl\(_2\) to set free [Ca\(^{2+}\)] from pCa 9.89 to pCa 4 and a protease inhibitor mix at 37°C for 20 min. The Ca\(^{2+}\)/EGTA ratio was calculated using the computer program described by Fabiato and Fabiato [38]. The binding mix was diluted with 5 ml of ice-cold washing buffer containing 25 mM Tris/HCl, pH 8.0, and 250 mM KCl and immediately filtered through Whatman GF/B filters presoaked with 1% polyethyleneimine. The filters were washed three times, and the radioactivity associated with the filters was determined by liquid scintillation counting. Non-specific binding was determined by measuring \( ^{3}H \)ryanodine binding in the presence of 50 \( \mu \)M unlabelled ryanodine. All binding assays were performed in duplicate.

**Sucrose density-gradient purification of recombinant RyR2 proteins**

RyR2 WT or the G230C mutant cell lysates (2.5 ml) layered on top of a 10.5 ml (7.5–25%, WT/WT) linear sucrose gradient containing 25 mM Tris, 50 mM Hepes, pH 7.4, 0.3 M NaCl, 0.1 mM CaCl\(_2\), 0.3 mM EGTA, 0.25 mM PMSF, 4 \( \mu \)g/ml leupeptin, 5 mM DTT, 0.3% CHAPS and 0.16% synthetic phosphatidylcholine. The gradient was centrifuged at 29000 rev./min in a Beckman SW-41 rotor at 4°C for 17 h. Fractions with a relatively small volume (0.7 ml/each) were collected to maximize the concentration of the RyR protein and, at the same time, to minimize the loss of the RyR2 protein after pooling the selected fractions. Peak fractions containing RyR proteins, as determined by immunoblotting, were pooled, aliquoted (60 ml) and stored at -80°C.

**Single-channel recordings**

Recombinant RyR2 WT and G230C mutant channels were purified from cell lysates prepared from HEK-293 cells transfected with the RyR2 WT or the G230C mutant cDNA by sucrose density-gradient centrifugation as described previously [24,37]. Heart phosphatidylethanolamine (50%) and brain phosphatidylserine (50%) (Avanti Polar Lipids), dissolved in chloroform, were combined and dried under nitrogen gas and resuspended in 30 \( \mu \)l of n-decane at a concentration of 12 mg of lipid per ml. Bilayers were formed across a 250 \( \mu \)m hole in a Delrin partition separating two chambers. The trans chamber (800 \( \mu \)l) was connected to the head stage input of an Axopatch 200A amplifier (Axon Instruments). The cis chamber (1.2 ml) was held at virtual ground. A symmetrical solution containing 250 mM KCl and 25 mM Hepes (pH 7.4) was used for all recordings, unless indicated otherwise. A 4-\( \mu \)l aliquot (\( \approx 1 \) \( \mu \)g of protein) of the sucrose density-gradient-purified recombinant RyR2 WT or the G230C mutant channels was added to the cis chamber. Spontaneous channel activity was always tested for sensitivity to EGTA and Ca\(^{2+}\). The chamber to which the addition of EGTA inhibited the activity of the incorporated channel presumably corresponds to the cytosolic side of the Ca\(^{2+}\)-release channel. The direction of single-channel currents was always measured from the luminal to the cytosolic side of the channel, unless mentioned otherwise. Recordings were filtered at 2500 Hz. Data analyses were carried out using the pclamp 8.1 software package (Axon Instruments). Free Ca\(^{2+}\) concentrations were calculated using the computer program described by Fabiato and Fabiato [38].

**Purification of RyR2ABC mutant protein**

Mouse RyR2 protein (amino acids 1–547) was cloned into a modified pET28 vector, and expressed in *Escherichia coli* Rosetta (DE3) pLacI cells (Novagen) as described previously for other RyR2 constructs [39,40]. The G230C mutation was introduced by the QuikChange® protocol (Stratagene). For the G230C mutant, cells were lysed by sonication in buffer A (250 mM KCl and 10 mM phosphate buffer, pH 8.5) with 25 \( \mu \)g/ml DNase I, 25 \( \mu \)g/ml lysozyme, 14 mM bME (2-mercaptoethanol) and 1 mM PMSF. The lysate was applied to a 25 ml Poros MC column (Tosoh Bioscience), washed with five CVs (column volumes) of buffer A and five CVs of buffer A plus 2% (v/v) buffer B (250 mM KCl and 500 mM imidazole, pH 8.5) and eluted with 60% (v/v) buffer B. The protein was dialysed against buffer A plus 14 mM bME and was cleaved simultaneously with recombinant TEV (tobacco etch virus) protease. The protein was then applied to a 25 ml
amylase column (New England Biolabs), washed with 10 CVs of buffer A plus 14 mM bME and eluted with buffer C (buffer A plus 10 mM maltose and 14 mM bME). The protein was applied again to the 25 ml Poros MC column in buffer A and then run on a HiLoad 16/10 Phenyl Sepharose HP column (GE Healthcare) in buffer D (1 M KCl, 10 mM Tris, pH 8.8, and 14 mM bME) and eluted with a gradient of 0–50% buffer E (50 mM KCl, 10 mM Tris/HCl, pH 8.8, and 14 mM bME). The protein was subsequently applied to a HiLoad Q-Sepharose HP column (GE Healthcare) with buffer E and eluted with a gradient of 0–50% buffer D. Then the protein was applied again to the 25 ml amylase column. Lastly, the protein was run on a HiLoad 16/60 Superdex 200 prep grade gel filtration column (GE Healthcare) in buffer consisting of 250 mM KCl, 10 mM Hepes, pH 7.4, and 14 mM bME. The protein was concentrated to 0.5 mg/ml using Amicon concentrators (3 kDa molecular mass cut-off; Millipore) and used fresh. The WT RyR2 (amino acids 1–547) protein was purified in a similar manner with a few differences: buffers A and B consisted of 10 mM Hepes, pH 7.4, or 500 mM imidazole, pH 7.4 rather than pH 8.5; the protein was applied to the amylase column only once, before TEV cleavage; and the HiLoad Q-Sepharose HP column was used before the HiLoad 16/10 Phenyl Sepharose HP column, both with buffers consisting of 10 mM Tris, pH 8.0 instead of pH 8.8.

Thermal melt analysis

The protein melting curves were measured by means of thermofluor experiments [41] as described previously for other RyR constructs [39,40,42,43]. Briefly, samples for melting curves contained 50 μl of purified protein at 0.1 mg/ml and SYPRO Orange solution (Invitrogen) using the manufacturer’s instructions in buffer consisting of 250 mM KCl, 10 mM Hepes, pH 7.4, and 14 mM bME. The melts were obtained in a DNA engine opticon 2 real-time PCR machine (Bio-Rad Laboratories) using the SYBR Green filter option. The temperature was changed from 20° to 95° C in 0.5° C steps. At every step, the temperature was kept constant for 15 s. The melting temperatures were obtained by averaging the peak points in the first derivatives of the melt curves from three replicate runs.

RyR2 homology model

A homology model for mouse RyR2 (amino acids 1–547) was produced using Modeller [44]. The structure of rabbit RyR1 (amino acids 1–559) was used as a template (PDB code 2XOA). An alignment between both sequences was produced using ClustalW.

Statistical analysis

All values shown are means ± S.E.M. unless indicated otherwise. To test for differences between groups, we used unpaired Student’s t tests (two-tailed). A P value <0.05 was considered to be statistically significant.

RESULTS

The RyR2-G230C mutation increases the propensity for SOICR

It has recently been suggested that the RyR2 mutation G230C causes CPVT via a SOICR-independent mechanism [32]. However, this hypothesis has not been tested directly. To assess whether this CPVT-linked RyR2 mutation G230C alters the propensity for SOICR, we generated a stable inducible HEK-293 cell line expressing the RyR2 WT or G230C mutant. These HEK-293 cells were perfused with increasing extracellular Ca2+ (0–2.0 mM) to induce SOICR. Fura-2 ratios of representative RyR2 WT (A) and G230C (B) cells were recorded using epifluorescence imaging. (C) The percentages of RyR2 WT (n=358) and G230C (n=390) cells that display Ca2+ oscillations at various extracellular Ca2+ concentrations. The results shown are means ± S.E.M. (n=7) (P<0.001 compared with WT). (D) Immunoblotting of RyR2 WT and the G230C mutant from the same amount of cell lysates using the anti-RyR antibody (34c) (n=3).

The RyR2-G230C mutation reduces the SOICR threshold

It has been shown that SOICR occurs when the store Ca2+ content reaches a threshold level, known as the SOICR threshold [20–24,28,45]. To determine the impact of the RyR2-G230C mutation on the threshold for SOICR, we monitored the ER luminal Ca2+ dynamics in HEK-293 cells using the FRET-based
ER luminal Ca\(^{2+}\) dynamics in G230C mutant channels during SOICR is shown in Figure 2(B). The G230C mutation significantly reduced the SOICR activation threshold (84% compared with 93% in WT) (P < 0.05). This is consistent with its enhanced SOICR propensity. Furthermore, the G230C mutation also reduced the SOICR termination threshold (45% compared with 59% in WT) (P < 0.05). The fractional Ca\(^{2+}\) release during SOICR (activation threshold − termination threshold) in RyR2 WT (35%) and G230C mutant (39%) cells was also significantly different (P < 0.05) (Figure 2E). There was no significant difference in the store capacity (F\(_{\text{max}}\) − F\(_{\text{min}}\)) between RyR2 WT and G230C mutant cells (Figure 2F). It should be noted that SOICR did not occur in control HEK-293 cells expressing no RyR2 and that SOICR was not affected by the IP3R (Inositol trisphosphate receptor) inhibitor xestospongin C [45], indicating that SOICR is mediated by RyR2. Collectively, these data indicate that the G230C mutation, like other CPVT RyR2 mutations that we have characterized previously [28,45], enhances the propensity for SOICR by reducing the SOICR threshold.

Figure 2 The G230C mutation decreases the threshold for SOICR in HEK-293 cells

Stable inducible HEK-293 cell lines expressing RyR2 WT or RyR2-G230C were transfected with the FRET-based ER luminal Ca\(^{2+}\)-sensing protein D1ER 48 h before single-cell FRET imaging. The expression of RyR2 WT and G230C was induced 24 h before imaging. The cells were perfused with KRH buffer containing increasing levels of extracellular Ca\(^{2+}\) (0–2 mM) to induce SOICR. This was followed by the addition of 1.0 mM tetracaine to inhibit SOICR, and then 20 mM caffeine to deplete the ER Ca\(^{2+}\) stores. FRET recordings from representative RyR2 WT (A) and G230C (B) cells (n = 53–84) are shown. To minimize the influence by CFP/YFP cross-talk, we used relative FRET measurements for calculating the activation threshold (C) and termination threshold (D) using the equations shown in (A). F\(_{\text{SOICR}}\) indicates the FRET level at which SOICR occurs, whereas F\(_{\text{termi}}\) represents the FRET level at which SOICR terminates. The fractional Ca\(^{2+}\) release (E) was calculated by subtracting the termination threshold from the activation threshold. The maximum FRET signal F\(_{\text{max}}\) is defined as the FRET level after tetracaine treatment. The minimum FRET signal F\(_{\text{min}}\) is defined as the FRET level after caffeine treatment. The store capacity (F) was calculated by subtracting F\(_{\text{termi}}\) from F\(_{\text{max}}\). The results shown are means ± S.E.M. (n = 5–6) (*P < 0.05 compared with WT).

The RyR2-G230C mutation enhances the luminal Ca\(^{2+}\) activation of single RyR2 channels

We have shown previously that a number of CPVT-linked RyR2 mutations reduce the SOICR threshold by enhancing the luminal Ca\(^{2+}\) activation of RyR2 [23,24]. To assess how the G230C mutation reduces the threshold for SOICR, we next determined the effect of the G230C mutation on the sensitivity of single RyR2 channels to luminal Ca\(^{2+}\) activation. Single RyR2 WT or mutant channels incorporated into lipid bilayers were first inactivated by the addition of EGTA. The cytosolic Ca\(^{2+}\) concentration was kept at ∼ 45 nM. The luminal Ca\(^{2+}\) concentration was then increased stepwise from 45 nM to 50 mM by additions of aliquots (1–12 ml) of CaCl\(_{2}\). As shown in Figure 3, in the near absence of cytosolic Ca\(^{2+}\) (45 nM), single RyR2 WT channels were barely activated by luminal Ca\(^{2+}\) concentrations of less than 10 mM (Figures 3A and 3C). However, under the same conditions, single RyR2-G230C mutant channels were activated by luminal Ca\(^{2+}\) at ∼ 300 μM (Figures 3B and 3C). Thus the G230C mutation markedly increases the sensitivity of single RyR2 channels to activation by luminal Ca\(^{2+}\).

Effect of the RyR2-G230C mutation on the sensitivity of RyR2 to cytosolic Ca\(^{2+}\) activation

We have also shown previously that CPVT-linked RyR2 mutations have little or no effect on the cytosolic Ca\(^{2+}\) activation of RyR2 [23,24]. To determine whether the G230C mutation alters the cytosolic Ca\(^{2+}\) activation of RyR2, we performed single-channel analysis of the RyR2 WT and the G230C mutant in the presence of various cytosolic Ca\(^{2+}\) concentrations (45 nM–100 μM) and in the near absence of luminal Ca\(^{2+}\) (∼ 45 nM). As shown in Figure 4, single RyR2 WT channels were activated by cytosolic Ca\(^{2+}\) with an EC\(_{50}\) of ∼ 330 nM. On the other hand, single G230C mutant channels were activated by cytosolic Ca\(^{2+}\) with an EC\(_{50}\) of ∼ 150 nM. We also performed \(^{[3H]}\)ryanodine-binding assays to assess the sensitivity of Ca\(^{2+}\) activation of the RyR2 WT and mutant channels. We found that the G230C mutation significantly reduced the EC\(_{50}\) of Ca\(^{2+}\) activation of \(^{[3H]}\)ryanodine binding to RyR2 (0.26 ± 0.05 μM compared with 0.50 ± 0.09 μM, G230C against WT) (n = 6, P < 0.05) (Figure 5). Thus the G230C mutation enhances both the cytosolic and luminal Ca\(^{2+}\) activation of RyR2.
Figure 3 The G230C mutation increases the sensitivity of single RyR2 channels to luminal Ca$^{2+}$ activation

Single-channel activities of RyR2 WT (A) and G230C (B) were recorded in a symmetrical recording solution containing 250 mM KCl and 25 mM Hepes (pH 7.4). EGTA was added to either the cis or trans chamber to determine the orientation of the incorporated channel. The side of the channel to which an addition of EGTA inhibited the activity of the incorporated channel presumably corresponds to the cytoplasmic face. The Ca$^{2+}$ concentration on both the cytoplasmic and the luminal face of the channel was adjusted to $\sim 45$ nM. The luminal Ca$^{2+}$ concentration was then increased to various levels by an addition of aliquots (1–12 μl) of CaCl$_2$ solution. Recording potentials, $-20$ mV. Openings downward and baselines indicated (short bars). Open probability (Po), mean open time (To) and mean closed time (Tc) are shown. The relationships between Po and luminal Ca$^{2+}$ concentrations (pCa) of single RyR2 WT (open circles) and G230C (filled circle) mutant channels are shown in (C). The data points shown are means ± S.E.M. from eight RyR2 WT and six G230C single channels (* P < 0.05 compared with WT).

The G230C mutation exerts an intrinsic structural effect on the N-terminal region of RyR2

The 3D crystal structure of the N-terminal region (resides 1–559) of RyR1 has been solved (PDB code 2XOA) [39,42,47]. This N-terminal region has been docked to an area near the four-fold symmetry axis on the cytoplasmic face of the 3D structure of the channel that is known to undergo large conformational changes during gating [43,48]. To gain some structural insights into the impact of the G230C mutation, we constructed a homology model of the corresponding region of RyR2 using the 3D structure of the N-terminal region of RyR1 as a template (Figure 6A). Gly$^{230}$ is located near the end of the linker that connects domains A and B, and is part of a conserved Gly-Gly motif that forms a tight turn just before the first $\beta$-strand of domain B (Figure 6B). In addition, the residue is completely buried near the interface between domains A and B. Recent modelling of the N-terminal region in the open and closed state of RyR1 has shown that this area undergoes large conformational changes during opening and closing [43]. Structures of disease mutant versions have also shown that proper domain–domain interactions are crucial for normal channel function and that a domain misalignment can facilitate channel opening. Because of the location of G230C within the linker connecting domains A and B, it is possible that it affects the relative orientation of these two domains, and thus the folding or stability of the N-terminal domains of the channel. To test this possibility, we expressed and purified the WT and the G230C mutant forms of the RyR2 N-terminal region.

Figure 4 The G230C mutation increases the sensitivity of single RyR2 channels to cytosolic Ca$^{2+}$ activation

Single-channel activities of the RyR2 WT (A) and G230C mutant (B) were recorded in the presence of $\sim 45$ nM luminal Ca$^{2+}$ and various concentrations of cytosolic Ca$^{2+}$. The relationships between open probability (Po) and cytosolic Ca$^{2+}$ concentrations (pCa) of single RyR2 WT (open circles) and G230C (filled circle) mutant channels are shown. The data points shown are means ± S.E.M. from seven single RyR2 WT and nine G230C mutant channels (*P < 0.05 compared with WT).

Figure 5 The G230C mutation increases the Ca$^{2+}$-dependent activation of [3H]ryanodine binding to RyR2

[3H]Ryanodine binding to cell lysate prepared from HEK-293 cells expressing the RyR2 WT or the G230C mutant was carried out at various Ca$^{2+}$ concentrations ($\sim 0.2$ nM–0.1 mM), 100 mM KCl and 5 nM [3H]ryanodine. Amounts of [3H]ryanodine binding at various Ca$^{2+}$ concentrations were normalized to the maximal binding (100%). The data points shown are means ± S.E.M. from six separate experiments (*P < 0.05 compared with WT).

In both cases, the final purified product yielded a single peak corresponding to a monomer in a size-exclusion chromatogram. We next measured the thermal stability through a thermofluor assay [39,40,43]. Both constructs appear to unfold in a two-step process upon heating (Figure 6C). The G230C mutation causes a leftward shift in the thermal stability of the first unfolding step ($42.0 \pm 0.5 ^\circ C$ for RyR2 WT, $39.7 \pm 0.3 ^\circ C$ for G230C). The decreased thermal stability suggests that the G230C mutation has...
together with those from previous studies support the view that enhanced SOICR is a common mechanism underlying RyR2-associated CPVT.

An important question then is how the G230C mutation enhances SOICR. Since SOICR results from RyR2 opening when the SR luminal Ca$^{2+}$ content exceeds a threshold level [20–22], it is reasonable to propose that the mutation may alter the response of RyR2 to luminal Ca$^{2+}$. Consistent with this hypothesis, we found that, similar to other CPVT RyR2 mutations we have tested previously [23,24], the G230C mutation sensitizes the channel to luminal Ca$^{2+}$ activation. In contrast, Marks and co-workers did not detect any significant impact of the G230C mutation on the luminal Ca$^{2+}$ activation of RyR2 [32]. The exact reason for this controversy is unknown at present. Differences in experimental conditions are likely to be involved. For example, we isolated recombinant RyR2 WT and mutant channels from transfected HEK-293 cells for single-channel recordings 24 h after transfection, whereas Meli et al. [32] collected HEK-293 cells 48 h after transfection. In the present study, we used CHAPS-solubilized, sucrose gradient-purified RyR2 WT and mutant channels for single-channel recordings, whereas crude ER microsomes were used in the study of Meli et al. [32]. Additionally, we used heart phosphatidylethanolamine and brain phosphatidylserine (Avanti Polar Lipids) at a 1:1 ratio at a concentration of 12 mg/ml to form the planar lipid bilayers, whereas Meli et al. [32] used phosphatidylethanolamine and phosphatidylcholine at a 3:1 ratio at a concentration of 40 mg/ml. Furthermore, we used 250 mM K$^+$ as the charge carrier at −20 mV in the presence of ~45 nM cytosolic Ca$^{2+}$, whereas Meli et al. [32] used 53 mM Ba$^{2+}$ or 700 mM Cs$^+$ as the charge carrier at 0 mV in the presence of 150 nM cytosolic Ca$^{2+}$. Future comprehensive and detailed studies will be required to systematically sort out this controversy.

In addition to its stimulatory effect on luminal Ca$^{2+}$ activation, the G230C mutation also increases the sensitivity of RyR2 to cytosolic Ca$^{2+}$ activation. In agreement with this observation, we also found that the G230C mutation enhances the Ca$^{2+}$-dependent activation of [3H]ryanodine binding. Thus the G230C mutation sensitizes RyR2 to both luminal and cytosolic Ca$^{2+}$ activation, which is similar to the impact of the CPVT RyR2 mutations V2475F [30] and N4104K [23] reported previously. The increased sensitivity of RyR2 to luminal Ca$^{2+}$ would enhance the channel’s response to elevated SR Ca$^{2+}$ content during SR Ca$^{2+}$ overload, and thus the initiation of SOICR, whereas the increased sensitivity of RyR2 to cytosolic Ca$^{2+}$ would enhance the gain of CICR and thus the propagation of Ca$^{2+}$ waves via CICR. Since luminal and cytosolic Ca$^{2+}$ activation of RyR2 is likely to be interactive and interdependent, the enhanced luminal Ca$^{2+}$ sensitivity would sensitize the channel to cytosolic Ca$^{2+}$ activation for a given SR Ca$^{2+}$ concentration. Similarly, the enhanced cytosolic Ca$^{2+}$ sensitivity would increase luminal Ca$^{2+}$ activation of the channel for a given cytosolic Ca$^{2+}$ concentration. Therefore this mutation-induced sensitization of both luminal and cytosolic Ca$^{2+}$ activation of RyR2 would increase the propensity for spontaneous Ca$^{2+}$ waves, Ca$^{2+}$ wave-evoked DADs, triggered activities and CPVT.

The crystal structure of the N-terminal region of RyR has recently been solved and shown to contain three domains (A, B, and C) [42]. The Gly$^{230}$ residue is located at the interface between domains A and B. It has been shown that RyR channel opening results in allosteric movements within the N-terminal domains, whereby an inter-subunit interface formed by domains A and B from neighbouring subunits may be disrupted [43]. Mutations at the interfaces between domains A, B, and C can cause a relative domain reorientation and thus affect surrounding interfaces [43].

**DISCUSSION**

It is well recognized that spontaneous Ca$^{2+}$ waves occur in cardiomyocytes as a result of activation of the RyR2 channel upon SR Ca$^{2+}$ overload. These store overload-induced Ca$^{2+}$ waves (or SOICR) can lead to DAD-associated triggered activity, which is a major cause of CPVT [3,6,16–22]. We have shown previously that a number of CPVT-linked RyR2 mutations increase the propensity for SOICR. We have further shown that these CPVT RyR2 mutations reduce the threshold for SOICR by sensitizing the channel to luminal Ca$^{2+}$ activation [23,24]. On the basis of these observations, we have proposed that enhanced SOICR as a result of increased luminal Ca$^{2+}$ activation of RyR2 is a common defect of CPVT RyR2 mutations [23,24,28]. At variance with this proposal, Marks and co-workers have recently shown that a novel CPVT RyR2 mutation G230C increases the sensitivity of single RyR2 channels to cytosolic Ca$^{2+}$ activation, but has no effect on luminal Ca$^{2+}$ activation, and proposed that the G230C mutation causes CPVT in a SOICR-independent manner [32]. However, the impact of this mutation on SOICR was not assessed in their study. Given the critical role of SOICR in triggering CPVT, in the present study, we have determined the effect of the G230C mutation on SOICR. We found that G230C markedly increases the propensity for SOICR by reducing its threshold. These data

![Figure 6 The G230C mutation reduces the thermal stability of the N-terminal domains of RyR2](image-url)

**A** Homology model of the RyR2 N-terminal domains. Gly$^{230}$ is shown by a black sphere. **B** Close-up view showing the Gly$^{230}$ residue as part of the turn preceding the first $\beta$-strand of domain B ($\beta$13). **C** Representative thermal melting curves of the WT (open circles) and the G230C mutant (filled circles) N-terminal domains of RyR2 (RyR2ABC) ($n = 3$).

an intrinsic destabilizing effect on the N-terminal domains of the RyR2 channel.
Previously, thermal analysis of disease-causing mutations in the N-terminal region of RyR1 has shown that some of these N-terminal mutations cause a thermal destabilization, whereas others did not cause such an effect. It is important to note that all mutations located at the interfaces between the three domains within a single subunit either exerted a significant destabilization or caused relative domain reorientations. Mutations that affect neither the thermal stability nor the relative domain orientations of the N-terminal domains may instead affect other domain–domain interfaces that are not present in the crystal structures. The G230C mutation located at the A–B domains interface probably affects domain packing. In addition, the Gly\(^230\) residue is part of a conserved Gly-Gly motif that forms a turn prior to the first \(\beta\)-strand of domain B, and is likely to be required for the proper geometry of the turn, and possibly the proper relative orientation of domains A and B. To assess its role in structure stabilization, we determined the effect of G230C on the thermal stability of the purified N-terminal region of RyR2. We found that the G230C mutation decreases the thermal stability of the N-terminal domains, suggesting that G230C has a destabilizing effect on the N-terminal domains that have been shown to be important for channel gating and the termination of Ca\(^{2+}\) release [43,45,48].

It is of interest to note that patients with CPVT RyR2 mutations do not normally display abnormal cardiac structure or function at rest [1–3]. How could RyR2 mutations that alter the intrinsic properties of the channel (e.g. the sensitivity to luminal and/or cytosolic Ca\(^{2+}\) activation) have little or no effect on EC (excitation–contraction) coupling at rest? This is probably due to the unique auto-regulatory mechanism of RyR2-mediated SR Ca\(^{2+}\) release [49,50]. An enhanced luminal/cytosolic Ca\(^{2+}\) activation of RyR2 would result in increased SR Ca\(^{2+}\) release and decreased SR Ca\(^{2+}\) content. A decrease in SR Ca\(^{2+}\) content in turn would decrease the activity of RyR2. Therefore, because of this feedback regulation of SR Ca\(^{2+}\) release by the SR Ca\(^{2+}\) content (or SR auto-regulation), modest alterations in the activity of RyR2 would not have a sustained impact on EC coupling under normal conditions [50]. However, under conditions of SR Ca\(^{2+}\) overload such as exercise or stress, increased Ca\(^{2+}\) activation of RyR2 will enhance the propensity for SOICR, DADs and thus triggered arrhythmias.

In summary, we show in the present study that the recently identified CPVT-linked RyR2 mutation G230C sensitizes the channel to luminal and cytosolic Ca\(^{2+}\) activation and reduces the threshold for SOICR, supporting the proposal that enhanced SOICR is a common defect of CPVT RyR2 mutations.

**REFERENCES**


