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ORIGINAL RESEARCH ARTICLE

Insights Into the Pathogenesis of Catecholaminergic Polymorphic Ventricular Tachycardia From Engineered Human Heart Tissue

BACKGROUND: Modeling of human arrhythmias with induced pluripotent stem cell–derived cardiomyocytes has focused on single-cell phenotypes. However, arrhythmias are the emergent properties of cells assembled into tissues, and the impact of inherited arrhythmia mutations on tissue-level properties of human heart tissue has not been reported.

METHODS: Here, we report an optogenetically based, human engineered tissue model of catecholaminergic polymorphic ventricular tachycardia (CPVT), an inherited arrhythmia caused by mutation of the cardiac ryanodine channel and triggered by exercise. We developed a human induced pluripotent stem cell–derived cardiomyocyte–based platform to study the tissue-level properties of engineered human myocardium. We investigated pathogenic mechanisms in CPVT by combining this novel platform with genome editing.

RESULTS: In our model, CPVT tissues were vulnerable to developing reentrant rhythms when stimulated by rapid pacing and catecholamine, recapitulating hallmark features of the disease. These conditions elevated diastolic Ca^{2+} levels and increased temporal and spatial dispersion of Ca^{2+} wave speed, creating a vulnerable arrhythmia substrate. Using Cas9 genome editing, we pinpointed a single catecholamine-driven phosphorylation event, ryanodine receptor–serine 2814 phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II, that is required to unmask the arrhythmic potential of CPVT tissues.

CONCLUSIONS: Our study illuminates the molecular and cellular pathogenesis of CPVT and reveals a critical role of calmodulin-dependent protein kinase II–dependent reentry in the tissue-scale mechanism of this disease. We anticipate that this approach will be useful for modeling other inherited and acquired cardiac arrhythmias.

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Clinical Perspective

What Is New?

- Engineered heart tissue fabricated from human pluripotent stem cell–derived cardiomyocytes effectively modeled catecholaminergic polymorphic ventricular tachycardia (CPVT) caused by dominant mutations in the cardiac ryanodine receptor (RYR2), including induction of arrhythmias by conditions that simulate exercise.
- Using selective pharmacology and genome editing, we identified activation of Ca^{2+}/calmodulin-dependent protein kinase II and calmodulin-dependent protein kinase II–mediated phosphorylation of RYR2 at serine 2814 as critical events required to unmask the latent arrhythmic potential of CPVT-causing *RYR2* mutations, highlighting a molecular pathway that links β-adrenergic stimulation to arrhythmogenesis in this disease.

What Are the Clinical Implications?

- This study identifies calmodulin-dependent protein kinase II activation as a central event in the triggering of arrhythmias in patients with CPVT, suggesting that calmodulin-dependent protein kinase II inhibition will be an effective and translatable strategy to treat CPVT caused by *RYR2* mutations.
- Under exercise conditions, CPVT mutations create heterogeneities in cardiac impulse propagation that make the working myocardium vulnerable to reentrant arrhythmias initiated by triggered beats emanating from the His-Purkinje system.
- The human engineered heart tissue platform will be useful to dissect mechanisms and treatment responses in diverse forms of arrhythmia.

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited arrhythmia caused predominantly by autosomal dominant mutation of the gene encoding the cardiac ryanodine receptor (RYR2), the main intracellular calcium release channel of cardiomyocytes.[1] Typically, patients with CPVT are asymptomatic at rest but develop potentially lethal ventricular tachycardia during exercise or emotional distress (Figure 1A and Figure IA in the online-only Data Supplement). In wild-type (WT) cells, when the cardiac action potential opens the voltage sensitive L-type Ca^{2+} channel located in the plasma membrane, the resulting local influx of Ca^{2+} triggers release of Ca^{2+} from the sarcoplasmic reticulum via RYR2 (Figure 1B). The resulting increase in cytoplasmic Ca^{2+} leads to sarcomere contraction. As the cell enters diastole, RYR2 closes and cytosolic Ca^{2+} is pumped back into the sarcoplasmic reticulum. In cells carrying CPVT mutations, RYR2 releases more Ca^{2+} into the cytoplasm, resulting in elevated diastolic Ca^{2+}, which drives exchange of sodium and calcium through the plasma membrane via the sodium-calcium exchanger,[3] leading to afterdepolarizations that may trigger additional action potentials. The molecular mechanism by which catecholamine stimulation unmasks the arrhythmic nature of CPVT mutations is not known, although catecholamine-induced activation of Ca^{2+}-calmodulin–dependent protein kinase II (CaMKII) has been implicated.[4,5] The mechanisms by which RYR2 mutation and consequent cellular abnormalities such as afterdepolarizations cause the organ-level phenotype of ventricular tachycardia are also uncertain. One theory is that triggered activity in the His-Purkinje system initiates ventricular tachycardia.[6,7] However, in a mouse CPVT model, ventricular arrhythmias required the CPVT-causing mutation in working myocardium, in addition to the His-Purkinje system.[8] suggesting that CPVT-causing mutations produce a substrate for reentrant or focal arrhythmias in the working myocardium. Understanding how single-cell Ca^{2+} handling abnormalities lead to tissue, organ, and clinical manifestations of CPVT has been hampered by limitations of currently available disease models.

The advent of induced pluripotent stem cell (iPSC) technology and efficient methods to differentiate iPSCs to cardiomyocytes (iPSC-CMs) has created exciting opportunities to study inherited arrhythmias.[9] iPSC-CMs have been generated from patients with CPVT[5,10–13] and other inherited arrhythmias and have been shown to capture key features of these diseases, including abnormal action potential duration and drug responses.[9] However, current iPSC-CM models of arrhythmia have been limited to isolated cells or cell clusters, leaving a large gap in modeling clinical arrhythmias, which are the emergent properties of cells assembled into myocardial tissue.[14]

We[15–17] and others[18] have developed engineered cardiac microphysiological systems that induce stem cell–derived and primary cardiomyocytes to adopt native-like laminar tissue architecture, permitting tissue-level measurement of contractility. Here, we integrated an engineered cardiac tissue with optogenetics, optical mapping, and iPSC-CMs to create a human tissue model of CPVT. We used this model and Cas9-mediated genome editing to investigate the molecular and cellular mechanisms underlying exercise-induced ventricular tachycardia in this disease.

METHODS

The expanded Methods section in the online-only Data Supplement provides a full description of experimental procedures. The authors declare that all supporting data are available within the article and its online-only Data Supplement. Materials will be shared on reasonable request.

Human iPSCs and Differentiation of iPSC-CMs

Patients provided informed consent to participate in this study under protocols approved by the Boston Children's Hospital Institutional Review Board. Fibroblasts were cultured from

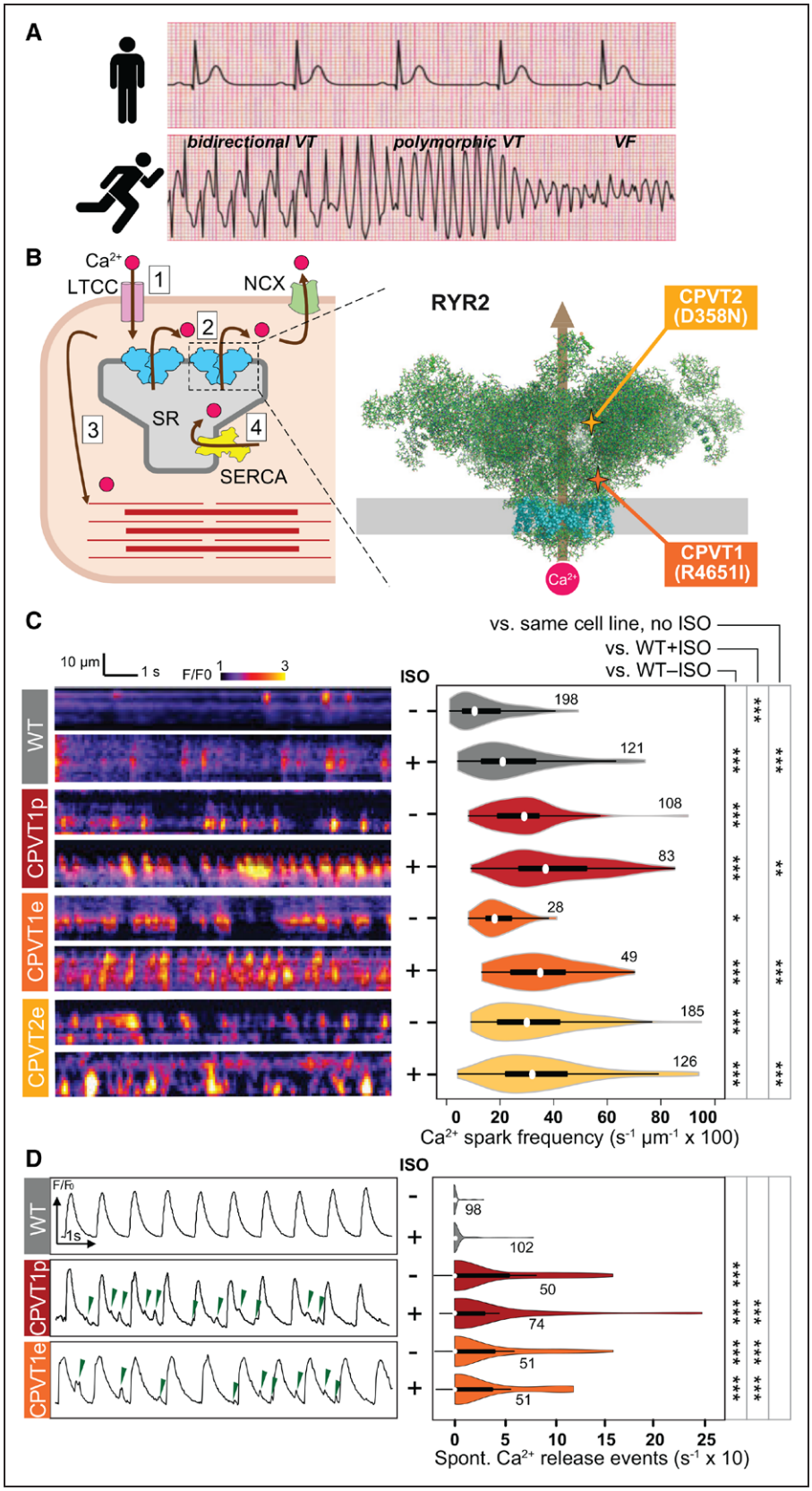

Figure 1. Characterization of Ca^{2+} oscillations in induced pluripotent cell–derived cardiomyocyte (iPSC-CM) clusters.
A, Patients with catecholaminergic polymorphic ventricular tachycardia (CPVT) have normal resting ECGs but severe, potentially life-threatening arrhythmias with exercise. Traces are idealized sketches shown for illustration purposes. **B**, CPVT pathophysiology. **Left**, Cartoon of cardiomyocyte Ca^{2+}-induced Ca^{2+} release. (1) Action potential opens L-type Ca^{2+} channel (LTCC). (2) Ca^{2+} induces opening of ryanodine receptor (RYR2) and release of Ca^{2+} from the sarcoplasmic reticulum (SR). (*Continued*)

Figure 1 Continued. (3) Elevated intracellular Ca^{2+} induces myofilament contraction. (4) Ca^{2+} is cleared from the cytosol by sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) and sodium-calcium exchanger (NCX). **Right**, CPVT mutations in RYR2 increase diastolic Ca^{2+} leak. Cartoon shows RYR2 structure based on CryoEM data.[2] Gray rectangle indicates the SR membrane. Transmembrane domains are shown in cyan. CPVT-causing RYR2 mutations in this study are highlighted. **C**, Incidence of Ca^{2+} sparks in quiescent iPSC-CMs. **Left**, Representative confocal line scans of Fluo-4 signal within individual iPSC-CMs in cell clusters. **Right**, Quantitative analysis. Number by each shape denotes number of cells examined. **D**, Incidence of spontaneous Ca^{2+} release events in spontaneously beating iPSC-CMs. **Left**, Representative Ca^{2+} signal traces, spatially averaged over confocal line scans within individual iPSC-CMs in cell clusters. Green arrowheads indicate spontaneous Ca^{2+} release events. **Right**, Quantitative analysis. Number by each shape denotes number of cells examined. Steel-Dwass nonparametric test with multiple-testing correction. ISO indicates isoproterenol; VF, ventricular fibrillation; VT, ventricular tachycardia; and WT, wild-type. **P*<0.05, ***P*<0.01, ****P*<0.001.

skin biopsies and reprogrammed by episomal transfection with reprogramming factors. iPSC lines were maintained in mTeSR1 medium (STEMCELL Technologies) and passaged in versene solution (15040066, Thermo Fisher Scientific) every 5 days on culture dishes precoated with Matrigel (hESC-Qualified Matrix, LDEV-Free, Corning) diluted 1:100.

The procedures for clustered regularly interspaced short palindromic repeat/Cas9 genome editing using a WT PGP1 human iPSC line containing doxycycline-inducible Cas9 were described in detail in a recent publication.[19] Guide RNA and homology-directed repair constructs are provided in the Methods in the online-only Data Supplement. We predicted Cas9-gRNA off-target sites using http://crispr.mit.edu/.[20] Amplicons containing the top 10 predicted sites were amplified from iPSCs and Sanger sequenced.

iPSCs differentiated to cardiomyocytes by modulating WNT signaling with CHIR99021 and IWR-1. iPSC-CMs were enriched by culture in lactate-containing media. Commercial human iPSC–derived cardiomyocytes (Cor4U; Axiogenesis, Cologne, Germany) were cultured according to the manufacturer's instructions.

Opto-Muscular Thin Film Construction

Micromolded gelatin was fabricated on glass coverslips such that the gelatin in the base region of muscular thin films (MTFs) would firmly attach to the glass coverslips but the gelatin in the cantilever region could be easily peeled. Cantilevers (1 mm wide×2 mm long) were laser engraved into the dehydrated micromolded gelatin. Gelatin chips were treated with ultraviolet-ozone for 90 seconds and rehydrated in a 2-mmol/L MES (2-(N-morpholino)ethanesulfonic acid) solution, pH 4.5, with 1 mg/ml collagen and 0.1 mg/mg fibronectin. After 2 hours at room temperature, the collagen and fibronectin solution was replaced with PBS. The gelatin chips were stored at 4°C until cell seeding.

Dissociated iPSC-CMs were plated on gelatin chips and then transduced with channelrhodopsin (ChR2) lentiviral vector, in which the cardiac troponin T promoter that drives ChR2-eYFPP was constructed on the basis of the FCK(1.3) GW plasmid with the cardiac troponin T promoter ChR2 and enhanced yellow fluorescent tag.[21,22]

Calcium Imaging of Cell Clusters

iPSC-CMs were seeded on Matrigel-coated glass bottom dishes for 5 days. After loading with Fluo-4 (F14201, Thermo Fisher Scientific) or Fluovolt (Thermo Fisher Scientific), cells were imaged with an Olympus FV1000 using line-scan mode (10 milliseconds per line, 1000 lines per recording). When indicated, cells were treated with protein kinase A (PKA) inhibitor (myristoylated 14-22 amide, 1 μmol/L) or CaMKII inhibitor (myristoylated autocamtide-2–related inhibitory peptide [AIP]). Isoproterenol and dantrolene were each used at 1 μmol/L.

Optical Recording of Opto-MTFs

Opto-MTFs were imaged using a modified tandem-lens macroscope (Scimedia) for simultaneous Ca^{2+} imaging and contractility measurement with optogenetic stimulation (Figure II in the online-only Data Supplement). Optogenetic stimulation was delivered with optical fibers mounted 500 μm above the gelatin chips.

Three days after transduction with ChR2 lentivirus, opto-MTFs were loaded with X-Rhod-1. Culture media was replaced with Tyrode solution and maintained at 37°C. When indicated, opto-MTFs were treated with 6 μmol/L dantrolene or 2 μmol/L AIP.

Opto-MTFs were stimulated with 10-millisecond optical pulses over a range of frequencies from 0.7 to 3 Hz with a custom LabVIEW program (National Instruments). For each recording, Ca^{2+} and dark field images were simultaneously acquired with 2000 and 400 frames at a frame rate of 200 and 100 Hz over 10 and 4 seconds, respectively.

Postprocessing of the raw calcium and dark-field imaging data was conducted with custom software written in MATLAB (MathWorks).

Statistical Analysis

Statistical analysis was performed with JMP Pro 14 (SAS Institute, Inc).

The significance between groups from cell cluster measurements was evaluated with the Steel-Dwass nonparametric test with multiple-testing correction. Statistical analysis of Western blots was performed with the Tukey-Kramer honestly significant difference test for multiple groups or Student *t* test for 2 groups.

For tissue-level differences, between-group differences in occurrence of reentry were evaluated with the Pearson χ^2 test and the Bonferroni correction for multiple comparisons with corrected *P* values by dividing *P* values by number of possible pairwise comparison tests (ie, 0.05/6, 0.01/6, 0.001/6 for 4 different tissues and 6 possible pairwise comparison tests; 0.05/3, 0.01/3, 0.001/3 for 3 different tissues and 3 possible pairwise comparison tests). Heterogeneities of calcium handling in the iPSC-CMs used in this study and neonatal rat ventricular myocytes or commercial iPSC-CMs were compared with 1-way ANOVA followed by the Tukey-Kramer honestly significant difference test. Tissue-level functional differences were tested with the Student *t* test corrected with Benjamini-Hochberg multiple-testing correction (false discovery rate, 20%).[23]

RESULTS

Ca^{2+} Handling in Individual iPSC-CM Within a Cell Cluster

We obtained skin fibroblasts from a patient with CPVT. This patient had a normal resting ECG but exercise-in-

duced bidirectional and polymorphic ventricular tachycardia (Figure IA in the online-only Data Supplement). Genotyping revealed that the patient had a heterozygous point mutation in RYR2 that caused substitution of isoleucine for arginine at position 4651 (R4651I; Figure 1B and Figure IB and IC in the online-only Data Supplement). Clinical genotyping did not implicate other candidate inherited arrhythmia genes. The fibroblasts were reprogrammed into iPSCs (line CPVT1p, where p indicates patient-derived; Figure IC). We used Cas9 genome editing[19] to introduce the patient mutation into a WT iPSC line, PGP1, yielding a CPVT line (PGP1-RYR-2R4651I, abbreviated CPVT1e, where e denotes engineered) otherwise isogenic to WT (Figure IB and ID in the online-only Data Supplement). In addition, we similarly used Cas9 genome editing to introduce a second CPVT-causing mutation, substitution of asparagine for aspartate at position 358 (D358N) into PGP1, yielding a second engineered CPVT line (PGP1-RYR2D358N, abbreviated CPVT2e; Figure 1B and Figure IB and III in the online-only Data Supplement). Sequencing of the top 10 predicted off-target genome editing sites for CPVT1e and CPVT2e did not identify unintended genome modification (Figure IV in the online-only Data Supplement). These iPSCs had normal karyotype and robustly differentiated into iPSC-CMs with comparable efficiency (Figure V in the online-only Data Supplement).

We analyzed Ca^{2+} handling of WT, CPVT1p, CPVT1e, and CPVT2e iPSC-CMs by loading spontaneously beating iPSC-CM clusters (3–10 cells per cluster) with the Ca^{2+}-sensitive dye Fluo-4 and performing confocal line-scan imaging within individual cells of a cluster. These clusters exhibited periods of spontaneous rhythmic beating interspersed with periods of quiescence. When recorded during periods of quiescence, both patient-derived and genome-edited isogenic CPVT1 iPSC-CMs had more frequent Ca^{2+} sparks than WT,[24] and this was further exacerbated by isoproterenol, a β-sympathomimetic (Figure 1C). The genome-edited isogenic CPVT2 iPSC-CM line also had more frequent Ca^{2+} sparks than WT, although this was not further exacerbated by isoproterenol (Figure 1C). During spontaneous beating, we observed spontaneous Ca^{2+} release events interspersed between Ca^{2+} transients (Figure 1D, left). Quantitative analysis showed greater frequency of spontaneous Ca^{2+} release events in beating CPVT iPSC-CMs, with and without isoproterenol stimulation (Figure 1D, right). These spontaneous Ca^{2+} release events correlated with membrane depolarization (Figure VH–VJ in the online-only Data Supplement), consistent with afterdepolarizations previously reported in CPVT iPSC-CMs and CMs.[4,5,12] These results confirm that both CPVT mutations tested are sufficient to increase Ca^{2+} release from RYR2, consistent with Ca^{2+} handling abnormalities reported in iPSC-CMs with other CPVT-causing RYR2 mutations.[5,10–13]

Opto-MTF–Engineered Heart Tissue Model of Inherited Arrhythmia

Because clinical arrhythmias emerge from the collective behavior of cardiomyocytes assembled into tissues, to better model inherited arrhythmias we integrated muscular thin films (MTFs),[25] optogenetics, and optical mapping to yield opto-MTFs, a platform that permits simultaneous assessment of myocardial Ca^{2+} transient propagation and contraction (Figure 2A and Figures II and VI in the online-only Data Supplement). iPSC-CMs were seeded onto 10×10-mm micromolded gelatin MTFs[16] (Figure VI in the online-only Data Supplement), so that they assembled into tissue with parallel cell alignment characteristic of native myocardium (Figure IIB and Movie I in the online-only Data Supplement). Lentivirus was used to program iPSC-CMs to express ChR2, a light-gated channel that allows cardiomyocytes to be paced with 488-nm light, as described previously.[25,26] ChR2 expression in cardiomyocytes was shown previously to alter minimally the electrophysiological properties of the tissue.[21] Using 10-millisecond pulses of 488-nm light, we optically stimulated an ≈0.79-mm^2 region, containing ≈500 cells, at 1 end of the opto-MTFs. This local activation stimulated Ca^{2+} transient propagation across the opto-MTFs in the direction of the long axis of muscle fibers. Propagation of Ca^{2+} transients was recorded with the Ca^{2+} sensitive fluorescent dye X-Rhod-1 at a resolution of 100×100 μm (Figure 2C and 2D and Figure VII and Movies II and III in the online-only Data Supplement). Ca^{2+} transient propagation into 2 film cantilevers at the far end of the opto-MTF induced iPSC-CM contraction, curving the cantilever and permitting measurement of mechanical systole (Figure 2C and 2D and Figure VII and Movies II and III in the online-only Data Supplement).[15–17] Each chip was composed of 3 individual MTFs (3×10 mm) that were isolated from one another, permitting independent assays within the same chip (Figure VIII and Movies IV and V in the online-only Data Supplement). Spatiotemporal characteristics of the opto-MTFs, including propagating Ca^{2+} transient activation time and propagation speed (CaS) and direction, were measured from the Ca^{2+} imaging data (Figure 2E). At a pacing rate of 1.5 Hz, the heterogeneity in CaS across space and time observed for the iPSC-CMs used in this study was comparable to or less than that observed with neonatal rat ventricular myocytes or commercial human stem cell–derived cardiomyocytes (Figure 2E and Figure IX in the online-only Data Supplement).

We used the opto-MTF platform to characterize the properties of CPVT iPSC-CMs assembled into engineered heart tissues. The spontaneous beating frequency of CPVT tissues was not different from that of WT tissues (Figure XA in the online-only Data Supplement). Spontaneous beats in both WT and CPVT originated

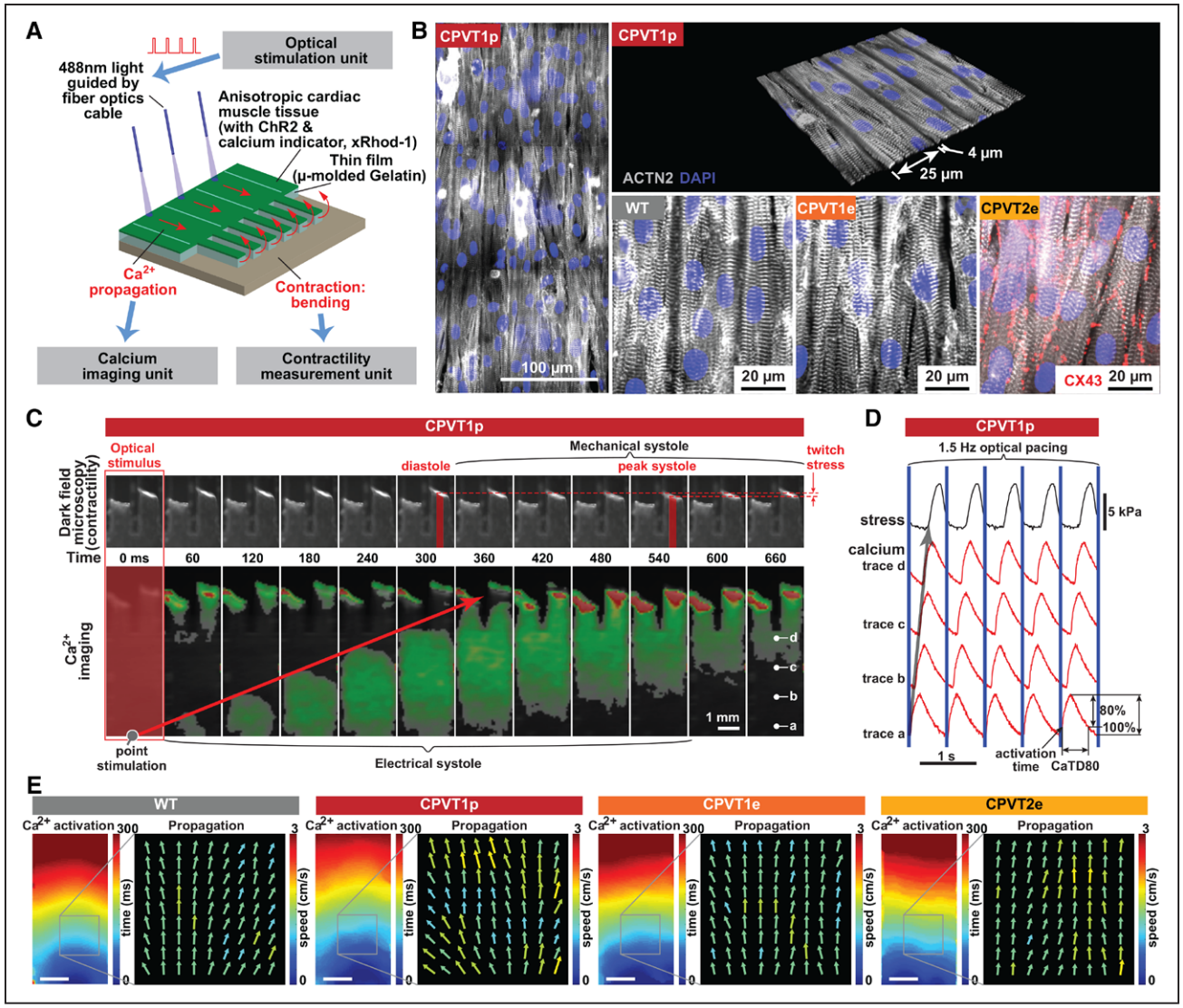

Figure 2. Opto-muscular thin film (MTF)–engineered heart tissue for arrhythmia modeling.
A, Schematic of opto-MTF system to optically pace and optically measure tissue-level Ca^{2+} wave propagation and contraction. Cardiomyocytes programmed to express channelrhodopsin (ChR2) are seeded onto micromolded gelatin with flexible cantilevers on 1 end. Focal illumination with optical fibers excites cells, resulting in Ca^{2+} wave propagation along the MTF and into the cantilevers. Ca^{2+} wave propagation is measured by fluorescent imaging of the Ca^{2+}-sensitive dye X-Rhod-1; mechanical contraction is assessed by dark-field imaging of the cantilevers. **B**, Confocal images of ACTN2-stained opto-MTFs. Micromolded gelatin induces induced pluripotent cell–derived cardiomyocytes to grow with their long axis aligned with the long axis of the MTF. **C**, Excitation-contraction coupling in CPVT1p opto-MTFs. Representative time-lapse images show Ca^{2+} wave propagation and mechanical systole recorded induced by optogenetic point stimulation. **D**, Ca^{2+} traces recorded at the points labeled a through d in the **right**-most image of **C**. Blue lines indicate optical pacing at the stimulation point. Activation time is the time to the maximal Ca^{2+} signal upstroke velocity. CaTD80 is the duration of the Ca^{2+} transient at 80% decay. **E**, Spatial maps of activation time and Ca^{2+} wave speed and direction for WT, CPVT1p, CPVT1e, and CPVT2e opto-MTFs at 1.5 Hz pacing, demonstrating well-ordered tissue behavior under these conditions. Bar, 1 mm. CPVT indicates catecholaminergic polymorphic ventricular tachycardia; and WT, wild-type.

from tissue edges, as expected from reduced source-to-load mismatch present at these sites. Whereas spontaneously beating, unstimulated CPVT iPSC-CMs in cell clusters composed of a few cells often exhibited Ca^{2+} transients with aberrant waveforms (Figure 1D), we did not observe any abnormal Ca^{2+} traces in spontaneously beating CPVT or control tissues recorded at lower resolution (Figure XB–XE in the online-only Data Supplement). These data indicate that Ca^{2+} transient abnormalities observed in single cells do not coordinate in spontaneously beating tissues to yield detectable abnormalities in tissue-level propagating Ca^{2+} transients (referred to hereafter in this study as calcium waves). This quiescent baseline behavior of spontaneously beating CPVT tissues parallels the normal baseline phenotype of patients with CPVT, who have few arrhythmias in the absence of exercise or emotional stress.

High Pacing Rate and β-Adrenergic Stimulation Induce Reentry in CPVT Opto-MTFs

Patients with CPVT are at risk for developing ventricular tachycardia during exercise or emotional stress. To

simulate key features of these provocative conditions in vitro, we treated opto-MTFs with increasing optical pacing frequency (1–3 Hz) or β-adrenergic stimulation (0–10 μmol/L isoproterenol). Remarkably, the 3 CPVT iPSC-CM lines but not WT opto-MTFs were vulnerable to spiral wave reentry, a cause of tachycardia at the tissue level (Figure 3A and 3B and Movies VI–IX in the online-only Data Supplement). The combination of both high pacing rate and isoproterenol most potently evoked reentry (Figure 3C). During reentry, the opto-MTF canti-

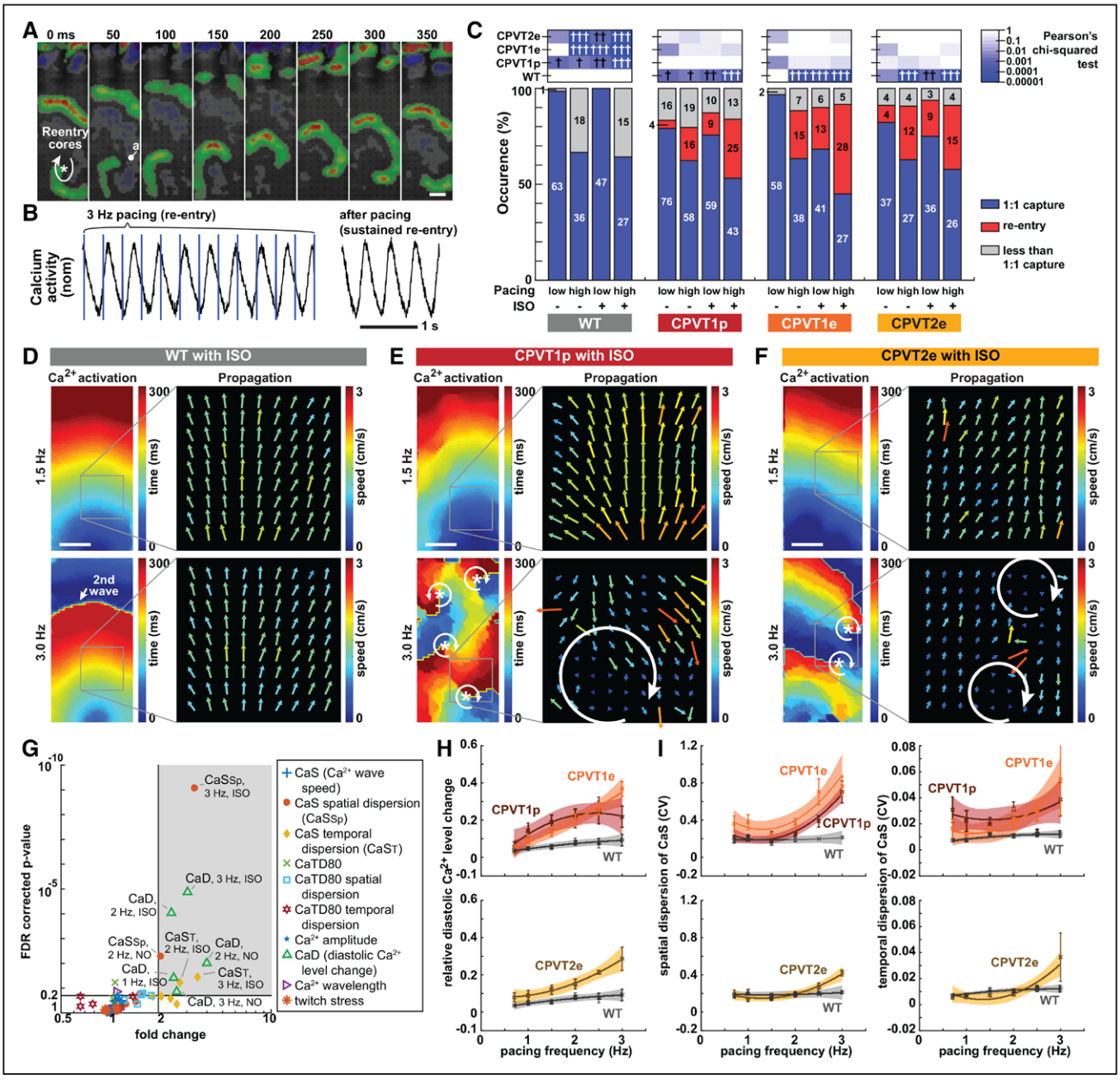

Figure 3. Characterization of catecholaminergic polymorphic ventricular tachycardia (CPVT) opto-muscular thin films (MTFs).
A, Time-lapse images of CPVT1e opto-MTF Ca^{2+} wave-front propagation. Ca^{2+} wave fronts, calculated from the temporal derivative of Ca^{2+} signals, show spiral wave reentry. **B**, Representative Ca^{2+} signal traces during reentry. Re-entrant Ca^{2+} signals were sustained even after pacing was discontinued. **C**, Occurrence of reentry in CPVT and wild-type (WT) opto-MTFs (high, ≥2-Hz pacing; low, <2-Hz pacing). High pacing rate and isoproterenol (ISO) increased reentry occurrence. Pearson χ^2 test was used with same pacing and ISO conditions. The Bonferroni correction for multiple comparisons was applied for 6 possible pairwise tests performed with a corrected *P* threshold. Bars are labeled with sample numbers. †*P*<0.05/6. ††*P*<0.01/6. †††*P*<0.001/6. **D** through **F**, Spatial maps of Ca^{2+} wave activation time and velocity in WT (**D**), CPVT1p (**E**), or CPVT2e (**F**) opto-MTFs. The same tissue is shown with 1.5- or 3-Hz pacing. Pacing at 3 Hz and ISO increased the spatiotemporal heterogeneity of CPVT tissues. **G**, Volcano plot shows 60 tissue-level parameters of Ca^{2+} propagation in WT vs CPVT1p and CPVT1e opto-MTFs. Each of the 10 markers represents the indicated property measured at 3 different pacing rates (1, 2, and 3 Hz) with and without ISO. Shaded region indicates parameters with false discovery rate–adjusted *P*<0.20 and >2-fold change. **H** and **I**, Relative diastolic Ca^{2+} level change from basal condition (**H**) and spatial and temporal dispersion of Ca^{2+} wave propagation speed (**I**) as a function of pacing frequency under ISO stimulation. Smooth lines are quadratic functions fit to the data. Shaded areas and error bars represent the 95% CI for the fit and SEM, respectively. Data from tissues with 1:1 capture were included in **G** through **I** (n=12 WT, 33 CPVT1p, 13 CPVT1e, 15 CPVT2e from >3 harvests). Bar in **A** and **D** through **F**, 1 mm. CaD indicates relative diastolic Ca^{2+} level change from basal condition; CaTD, the duration of the Ca^{2+} transient; and CaS, Ca^{2+} transient activation time and propagation speed.

levers generated less stress and induced asynchronous contraction (Figure XI and Movies VI, VII, and X in the online-only Data Supplement), mimicking the loss of forceful coordinated contraction that impairs cardiac output in clinical ventricular tachycardia. Dantrolene, a small molecule that inhibits release of Ca^{2+} through ryanodine receptors, inhibits CPVT arrhythmias.[27,28] Consistent with previous studies,[10,27] dantrolene reduced abnormal Ca^{2+} handling in CPVT iPSC-CMs (Figure XII in the online-only Data Supplement). Dantrolene likewise suppressed reentry in CPVT1e opto-MTFs (Figure XIII in the online-only Data Supplement). These data show that assembly of CPVT iPSC-CMs into opto-MTFs models key features of the disease at a tissue level. Furthermore, the data implicate reentry as an arrhythmia mechanism in CPVT.

To better understand the development of reentry in CPVT tissues, we analyzed optical mapping data of Ca^{2+} wave propagation. When challenged with 3-Hz pacing and isoproterenol, WT opto-MTFs retained organized Ca^{2+} waves, as shown by coherent Ca^{2+} activation maps and relatively homogeneous Ca^{2+} wave propagation speed and direction (Figure 3D). At 1.5-Hz pacing with or without isoproterenol, CPVT1p, CPVT1e, and CPVT2e opto-MTFs also were relatively coordinated (Figure 3E and 3F, top). However, rapid pacing of the same CPVT tissues induced reentry in which Ca^{2+} waves propagated in a circular pattern, associated with increased spatial dispersion of CaS (Figure 3E and 3F, bottom, and Figure XIV and Movie XI in the online-only Data Supplement).

To characterize the tissue-level mechanisms that make CPVT tissues vulnerable to reentry, we compared WT with CPVT1 tissues for 10 different parameters (CaS, spatial and temporal dispersion of CaS, Ca^{2+} transient duration at 80% recovery, spatial and temporal dispersion of Ca^{2+} transient duration at 80% recovery, relative diastolic Ca^{2+} level change from basal condition, Ca^{2+} wave amplitude, Ca^{2+} wavelength, and twitch stress) at 3 different pacing rates (1, 2, and 3 Hz) with and without isoproterenol (Figure 3G and Figure XV in the online-only Data Supplement). For these analyses of the conditions that made CPVT1 tissues vulnerable reentry substrates, we focused on recordings with 1:1 capture and without active reentry. Under higher pacing rates and isoproterenol stimulation, CPVT1 tissues differed from WT (>2-fold change, nominal $P<0.05$, and false discovery rate <0.2) in 3 parameters: relative diastolic Ca^{2+} level, spatial dispersion of CaS, and temporal dispersion of CaS. Specifically, significant differences were observed in relative diastolic Ca^{2+} level at 3 Hz±isoproterenol, 2 Hz±isoproterenol, and 1 Hz+isoproterenol; temporal dispersion of CaS at 3 Hz+isoproterenol and 2 Hz+isoproterenol; and spatial dispersion of CaS at 3 Hz+isoproterenol and 2 Hz–isoproterenol. We further analyzed the effect of pacing rate on these 3 parameters in isoproterenol-stimulated CPVT and WT tissue (Figure 3H and 3I and Figure XV in the online-only Data Supplement). In isoproterenol-stimulated WT tissues, diastolic Ca^{2+} levels and spatial and temporal dispersion of CaS were relatively insensitive to changes in pacing rate from 1 to 3 Hz. In contrast, as CPVT1p, CPVT1e, and CPVT2e tissues were paced at higher rates, they developed greater relative diastolic Ca^{2+} level and greater spatial and temporal dispersion of CaS (Figure 3H and 3I and Figure XV in the online-only Data Supplement). Dispersion of CaS was also manifested by irregular Ca^{2+} activation maps observed in CPVT tissue paced at 3 Hz (Movie XI and Figure XIV in the online-only Data Supplement. These irregular Ca^{2+} activation maps were not observed when the same tissues were paced at 2 Hz (Movie XII and Figure XIV in the online-only Data Supplement). Because heterogeneity increases tissue vulnerability to reentry,[14] this analysis indicates that rapid pacing and isoproterenol stimulation create a vulnerable arrhythmia substrate by increasing spatial and temporal heterogeneity in CaS. This increased tissue heterogeneity might be linked to pacing-dependent elevation of diastolic Ca^{2+}, which drives afterdepolarizations and impairs gap junction function,[29] thereby potentially reducing intercellular connectivity.

We next analyzed events that initiated reentry in the vulnerable CPVT substrate. We identified 8 recordings that captured the initiation of reentry (Table I in the online-only Data Supplement). The rare reentry events that we observed at low pacing rates (Figure 3C) were initiated by spontaneous Ca^{2+} waves originating from edges of the tissue that collided with pacing-driven Ca^{2+} waves (Movie X and Figure XVI in the online-only Data Supplement). High pacing rates suppressed these spontaneous Ca^{2+} waves, and reentry events were initiated by regional conduction block (Figure 4 and Movies XIII and XIV in the online-only Data Supplement). Isoproterenol-treated CPVT1p tissue paced at 2 Hz showed well-ordered Ca^{2+} wave propagation (Figure 4A and 4B). When the same tissue was paced at 3 Hz, there was greater dispersion of propagation velocity, including regions with functional conduction block (Figure 4C, gray areas in velocity vector field). Ca^{2+} waves propagating around the focal block reentered the original path, resulting in rotor formation. A similar example of reentry initiation with functional conduction block in CPVT1e and CPVT2e tissues is shown in Movies VIII, XV, and XVI and Figure XVII in the online-only Data Supplement. Although the mechanisms underlying functional, regional conduction block in CPVT tissue require further study, they were associated with elevated relative diastolic Ca^{2+} (Figure 3G and 3H), which could reduce iPSC-CM excitability[30] and cell-cell coupling.[29]

Together, our engineered tissue model demonstrates that CPVT tissue is vulnerable to circus movement with

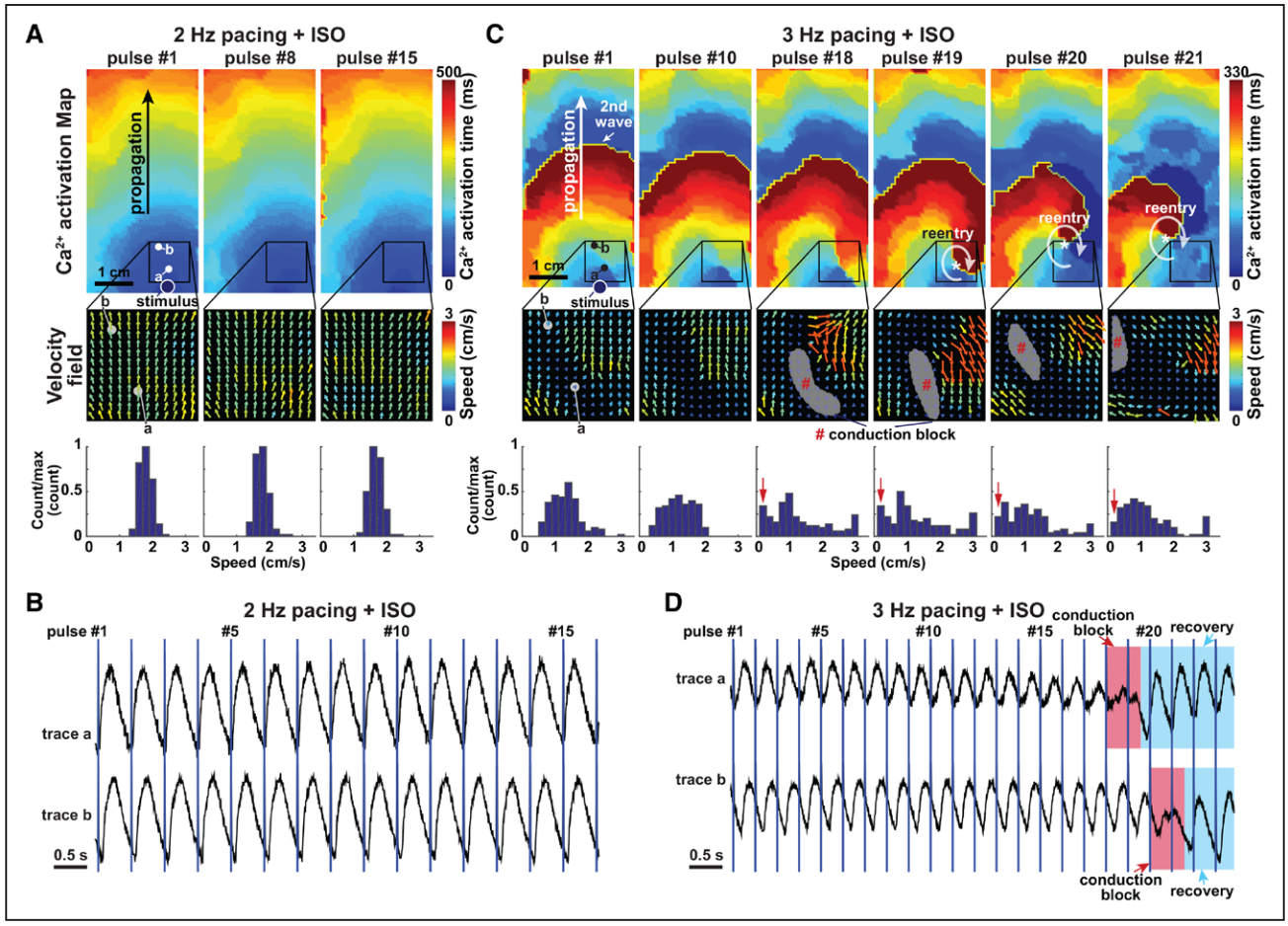

Figure 4. Initiation of reentry in catecholaminergic polymorphic ventricular tachycardia (CPVT) opto-muscular thin films (MTFs).
A, CPVT1p opto-MTF at 2-Hz pacing with isoproterenol (ISO). The Ca^{2+} activation map and velocity fields were well ordered. Speed histogram reflects narrow range of values. **B**, Ca^{2+} tracings from points a and b in **A**. **C**, The same opto-MTF as in **A** paced at 3 Hz with ISO. There are greater heterogeneity in the velocity field and disorganization of the Ca^{2+} activation map. Localized conduction block that permitted reentrant Ca^{2+} wave propagation becomes evident at pulses 18 and 19. Histograms indicate greater spatial dispersion of speed. **D**, Ca^{2+} tracings at points a and b in **C**. Red shading indicates conduction block overlying recording points; blue shading, recovery from block.

reentry. Rapid pacing and isoproterenol stimulation increased relative diastolic Ca^{2+} and spatiotemporal dispersion of CaS. In this context, localized conduction block initiated rotor formation.

CaMKII Phosphorylation of RYR2–Serine 2814 Is Required to Unmask CPVT Arrhythmic Potential

Although catecholamine stimulation is well known to provoke arrhythmia in CPVT, the molecular targets through which β-adrenergic stimulation unmasks the latent arrhythmic potential of RYR2 mutations were incompletely defined. β-Adrenergic stimulation activates numerous signaling pathways, including CaMKII and PKA (Figure 5A). When we inhibited PKA using a potent, cell-permeable peptide,[31] the incidence of Ca^{2+} sparks was not significantly reduced in CPVT1p iPSC-CMs (Figure 5B). In contrast, CaMKII inhibition with cell-permeable AIP (Figure XVIIIA in the online-only Data Supplement), a highly selective and potent CaMKII inhibitor,[32] strongly reduced the incidence of Ca^{2+} sparks and afterdepolarizations in CPVT1p, CPVT1e, and CPVT2e iPSC-CMs (Figure 5B and Figure XVIIIB and XVIIIC in the online-only Data Supplement). This effect is consistent with previous reports using a small-molecule inhibitor[4,5] and with a recently reported CaMKII-dependent, PKA-independent pathway that mediates arrhythmogenesis provoked by adrenergic stimulation.[33] To model the effect of CaMKII inhibition at the tissue level, we treated CPVT1 and CPVT2 opto-MTFs with AIP. AIP inhibited the development of reentry provoked by rapid pacing and isoproterenol (Figure 5C and Movie XVII in the online-only Data Supplement). Analysis of diastolic Ca^{2+} level (Figure 5D) and spatial and temporal dispersion of Ca^{2+} wave speed (Figure 5E) showed that these key parameters of CPVT tissue vulnerability were normalized by AIP. These data suggest that CaMKII is a key signaling molecule in the pathogenesis of CPVT.

CaMKII targets multiple proteins that directly or indirectly affect Ca^{2+} handling.[34] One important CaMKII target is serine 2814 (S2814) on RYR2 itself[35] (Figure 5A).

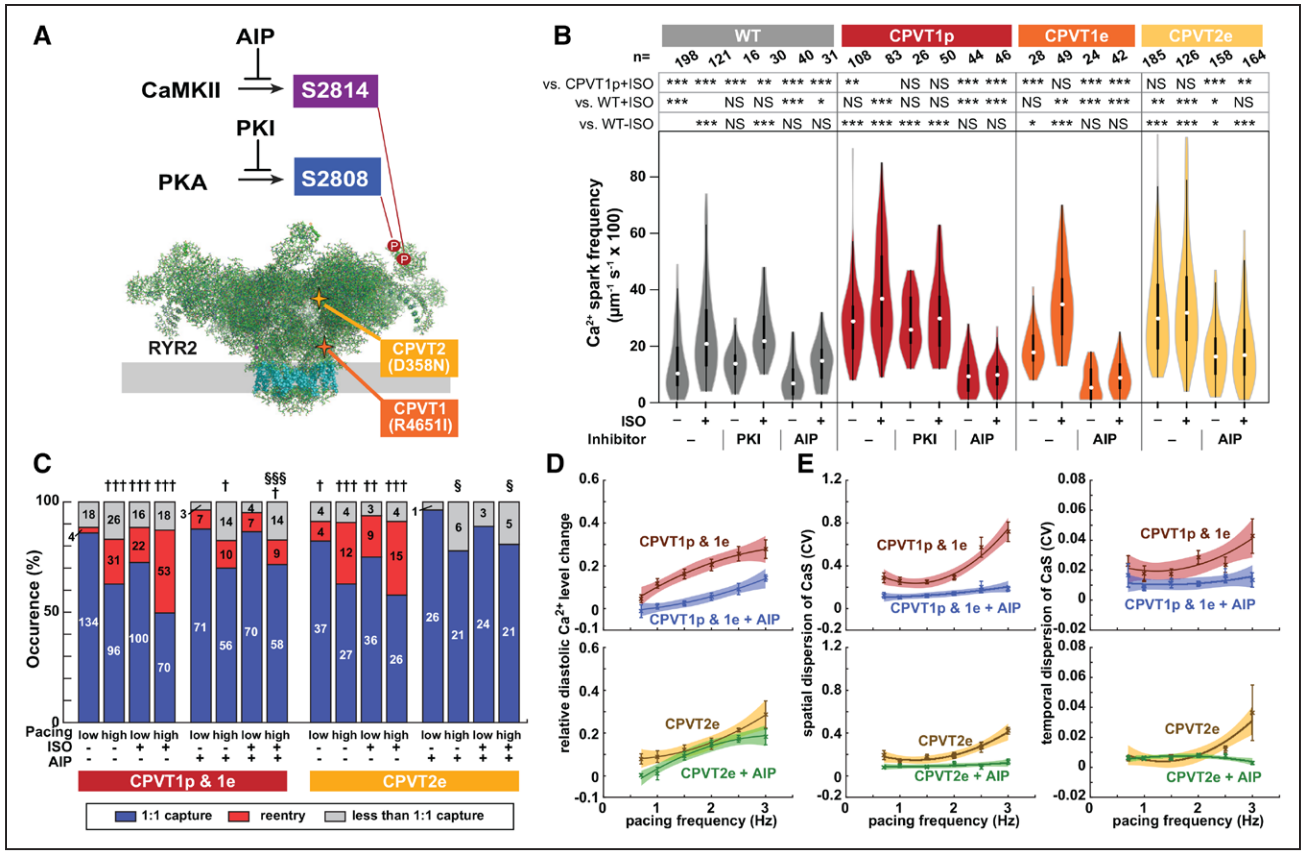

Figure 5. Ca^{2+}-calmodulin–dependent protein kinase II (CaMKII) inhibition suppresses the catecholaminergic polymorphic ventricular tachycardia (CPVT) arrhythmic phenotype.

A, Schematic of ryanodine receptor (RYR2; 2 subunits of tetramer shown). Three key residues are highlighted: S2808, the target of protein kinase A (PKA) phosphorylation; serine 2814 (S2814), the target of CaMKII phosphorylation; and R4651, mutated in CPVTp. **B**, Induced pluripotent stem cell–derived cardiomyocytes (iPSC-CMs) in isolated cell clusters were treated with isoproterenol (ISO) and selective CaMKII (autocamtide-2-related inhibitory peptide [AIP]) or PKA inhibitors (PKIs). Ca^{2+} sparks were imaged by confocal line scanning within individual iPSC-CMs. Data without inhibitors are the same as in Figure 1C. Steel-Dwass nonparametric test with multiple testing correction **P*<0.05, ***P*<0.01, ****P*<0.001. **C.** Occurrence of reentry in CPVT1 and CPVT2 engineered tissues treated with a CaMKII inhibitor (AIP). Bars are labeled with samples sizes. Pearson χ^2 test. †Versus wild-type (WT) with the same pacing and ISO conditions; §Versus CPVT with the same pacing and ISO conditions. The Bonferroni correction for multiple comparisons was applied for 3 possible pairwise tests performed with a corrected *P* threshold. †§*P*<0.05/3. ††*P*<0.01/3. †††§§§*P*<0.001/3. **D** and **E**, Relative diastolic Ca^{2+} level change from basal condition (**D**), and spatial and temporal dispersion of Ca^{2+} wave propagation speed (**E**) as a function of pacing frequency under ISO stimulation in CPVT1 and CPVT2 before and after AIP loading. Only tissues responding 1:1 to every stimulus were included in **D** and **E** (n=33 CPVT1p, 15 CPVT1p+AIP, 13 CPVT1e, 15 CPVT2e, and 9 CPVT2e+AIP from >3 harvests). NS indicates not significant.

RYR2-S2814 phosphorylation by CaMKII enhances diastolic RYR2 Ca^{2+} leak and is proarrhythmic.[36] To test the hypothesis that CaMKII-mediated phosphorylation of RYR2-S2814 is essential for expression of CPVT mutations, we used Cas9 genome editing to replace S2814 with alanine (S2814A) in RYR2 alleles in both RYR2 WT and $RYR2^{R4651I/+}$ backgrounds and showed that these mutations block this phosphorylation event (Figures IV and XIX in the online-only Data Supplement). We refer to these mutant alleles as WT-S2814A and CPVT1e-S2814A, respectively. RYR2 is also phosphorylated on S2808 by PKA,[37] and this phosphorylation event has been implicated in increasing RYR2 diastolic Ca^{2+} leak.[38] Therefore, we used genome editing to generate analogous RYR2-S2808A homozygous mutant lines, called WT-S2808A and CPVT1e-S2808A (Figures IV and XIX in the online-only Data Supplement). In keeping with the effect of CaMKII inhibitory peptide, in quiescent iPSC-CMs in cell clusters, the incidence of Ca^{2+} sparks in CPVT1e-S2814A was less than in CPVT1e and comparable to that in WT at baseline and with isoproterenol stimulation (Figure 6A and 6B). In contrast, the incidence of these events was similar in CPVT1e-S2808A and CPVT1e iPSC-CMs (Figure 6A and 6B), consistent with the ineffectiveness of pharmacological PKA inhibition (Figure 5B). Similar results were obtained from spontaneously beating cell clusters (Figure 6C and 6D). These data indicate that CaMKII phosphorylation of RYR2-S2814 is required to unmask the latent arrhythmic potential of the CPVT R4651I mutation.

We modeled the effect of ablation of the RYR2-S2814 CaMKII phosphorylation site on the development of reentry in CPVT engineered heart tissue. We fabricated opto-MTFs from CPVT1e-S2814A iPSC-CMs (Figure 6E). Unlike CPVT1p and CPVT1e tissues, rapid pacing and isoproterenol did not induce reentry in

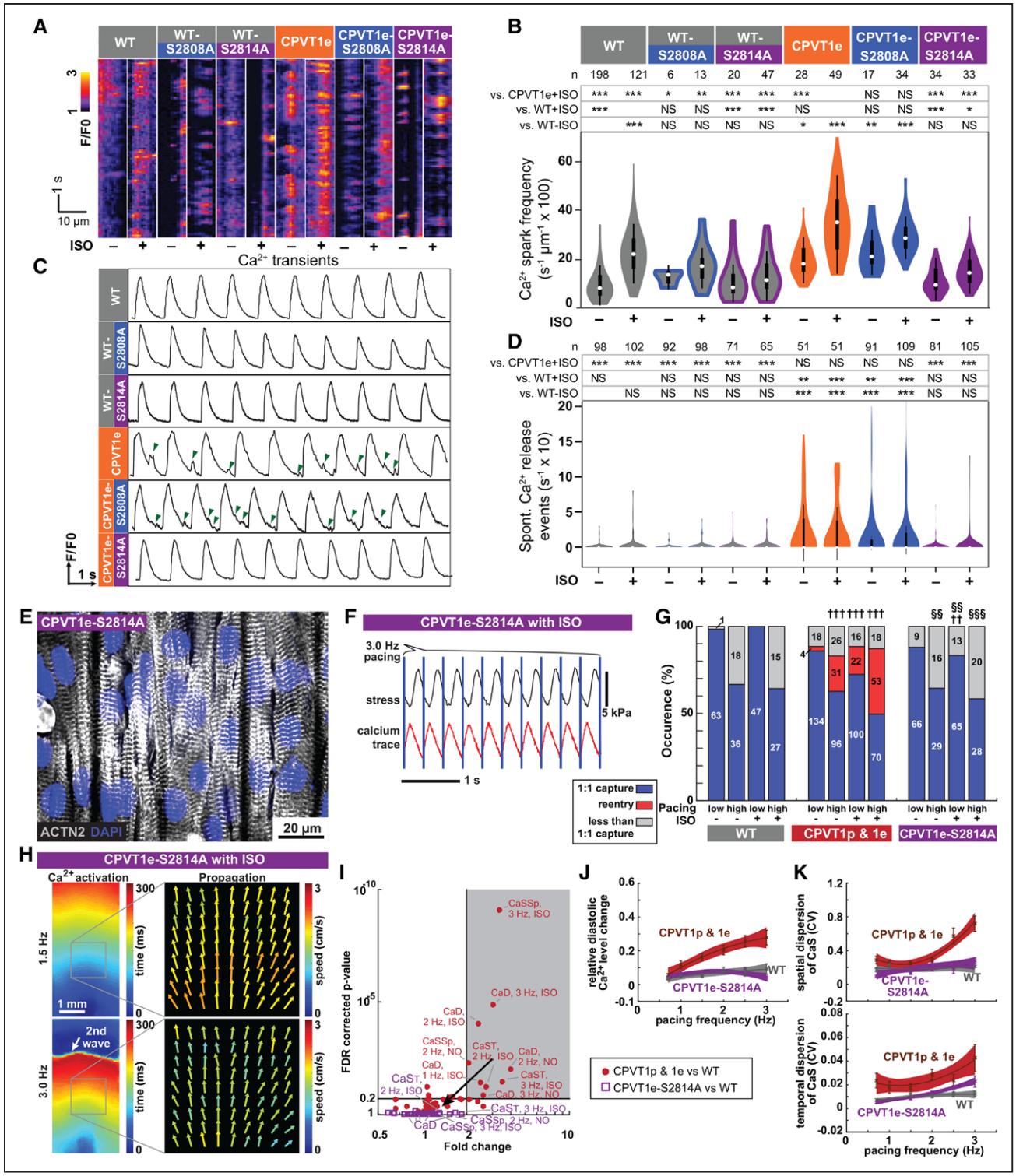

Figure 6. Ca^{2+}-calmodulin–dependent protein kinase II (CaMKII) phosphorylation of ryanodine receptor (RYR2)–serine 2814 (S2814) is required to unmask the catecholaminergic polymorphic ventricular tachycardia (CPVT) arrhythmic phenotype.

A and **B**, Incidence of Ca^{2+} sparks in quiescent induced pluripotent stem cell–derived cardiomyocytes (iPSC-CMs) in cell clusters. **A**, Representative traces. **B**, Quantitative analysis. Wild-type (WT) and CPVT1e data are the same as in Figure 5B. Steel-Dwass nonparametric test with multiple testing correction. *P<0.05. **P<0.01. ***P<0.001. **C** and **D**, Incidence of spontaneous Ca^{2+} release events in spontaneously beating iPSC-CMs in cell clusters. **C**, Representative traces. Green arrowheads indicate spontaneous Ca^{2+} release events. **D**, Quantitative analysis. WT and CPVT1e data are the same as in Figure 1D. Statistics and symbols as in **B**. **E**, Confocal image of opto-muscular thin film (opto-MTF) constructed with CPVT1e-S2814A iPSC-CMs. Myocytes are aligned by micromolded gelatin substrate. **F**, Representative CPVT1e-S2814A opto-MTF. Ca^{2+} transients and systolic contraction were coupled 1:1 with 3-Hz optical stimuli (blue lines). **G**, Occurrence of reentry in CPVT1e-S2814A compared with WT (††, †††) and CPVT1e (§§, §§§) opto-MTFs under matching conditions with the Pearson χ^2 test. The Bonferroni correction for multiple comparisons was applied for 3 possible pairwise tests performed with a corrected P threshold ††§§P<0.01/3. †††§§§P<0.001/3. Bars are labeled with sample numbers. **H**, Spatial maps of the same CPVT1e-S2814A opto-MTF paced at 1.5 or 3.0 Hz in the presence of isoproterenol (ISO). (*Continued*)

Figure 6 Continued. Ca^{2+} activation time and Ca^{2+} wave propagation speed were well organized and relatively homogeneous compared with CPVT1 (see Figure 3). **I**, Volcano plot of 60 tissue-level parameters of Ca^{2+} wave propagation (see Figure 3). Unlike CPVT1 tissue parameters, CPVT1e-S2814A tissue parameters were not statistically different from those of WT. **J** and **K**, Relative diastolic Ca^{2+} level change from basal condition (**J**), and spatial and temporal dispersion of Ca^{2+} wave propagation speed (**K**) as a function of pacing frequency under ISO stimulation in CPVT1e-S2814A compared with CPVT and WT. Smooth lines are quadratic functions fitted to the data; shaded areas and error bars show the 95% CI for the fit and SEM, respectively. Only tissues responding 1:1 to every stimulus were included in **I** to **K** (n=12 WT, 33 CPVT1p, 13 CPVT1e, 15 CPVT2e, and 18 CPVT1e-S2814A from >3 harvests). FDR indicates false discovery rate; and NS, not significant.

CPVT1e-S2814A tissues (Figure 6F and 6G and Movie XVIII in the online-only Data Supplement). These tissues retained well-ordered Ca^{2+} waves without regions of conduction block (Figure 6H), which we observed in CPVT1p, CPVT1e, and CPVT2e opto-MTFs under the same conditions (Figures 3 and 4). We analyzed Ca^{2+} wave propagation and other tissue properties of opto-MTFs between CPVT1e-S2814A and WT (purple, Figure 6I) and compared results with the previous analysis of the differences between CPVT and WT (Figure 3G, shown in red in Figure 6I). The RYR2-S2814A substitution did not alter global CaS (Figure XV in the online-only Data Supplement), but notably, it normalized the 3 parameters (relative diastolic Ca^{2+} level change from basal condition, spatial dispersion of CaS, and temporal dispersion of CaS) that were significantly different between CPVT and WT under higher pacing rates and isoproterenol. These 3 parameters became progressively higher in isoproterenol-treated CPVT than WT tissues with increasing pacing rate (Figure 3H and 3I and Figure XV in the online-only Data Supplement), but these abnormalities were abolished by the RYR2-S2814A substitution (Figure 6J and 6K and Figure XV in the online-only Data Supplement). These data show that preventing RYR2-S2814 phosphorylation normalizes pacing- and isoproterenol-induced CaS heterogeneity and relative diastolic Ca^{2+} level, resulting in a substrate that is less vulnerable to tissue-level reentry.

DISCUSSION

By integrating optogenetics, tissue engineering, lab-on-a-chip technology, and iPSC technology, we have created an engineered human tissue model to study arrhythmia mechanisms and to test antiarrhythmic therapy. We combined this platform with patient-derived and genome-edited iPSC-CMs to model the inherited arrhythmia CPVT at the tissue level. Similar strategies could be used to model other forms of inherited arrhythmia or to develop high-fidelity tissue-level models for cardiovascular drug toxicity studies.

Our engineered tissue model recapitulated key features of the clinical phenotype. First, under baseline conditions, the CPVT engineered tissues did not exhibit ectopic Ca^{2+} wave initiation, consistent with the normal baseline ECGs typically observed in patients at rest. This contrasts to the abnormal phenotype of unstimulated CPVT iPSC-CMs or dissociated murine CMs.[39] Second, adrenergic stimulation and rapid pacing induced reentrant rhythms in the CPVT engineered tissues, mimicking exercise-induced ventricular tachycardia, which is a hallmark of the clinical phenotype.

Our CPVT models provide insights into disease pathogenesis. Although it was previously known that adrenergic stimulation of CaMKII was important for arrhythmogenesis in CPVT,[4,5] our study identified a single CaMKII target site that must be phosphorylated to unmask the latent arrhythmic potential of the RYR2-R4651I CPVT mutation. This CaMKII target site is on RYR2 itself, at S2814. Phosphorylation of this site is known to increase RYR2 Ca^{2+} release and susceptibility to arrhythmia.[35,36] Our results provide a molecular pathway that explains how exercise provokes arrhythmia in patients with CPVT and suggest that inhibiting this pathway will protect patients from developing lethal arrhythmia. We demonstrated that CaMKII activity is required to unmask the abnormal phenotype of several independent CPVT-causing RYR2 mutations (see the accompanying study by Bezzerides et al[40]), although further work is required to demonstrate the precise requirement for RYR2-S2814 phosphorylation across multiple genotypes. The accompanying study by Bezzerides et al[40] further demonstrates that CaMKII inhibition by cardiac targeted gene therapy effectively suppresses ventricular arrhythmia in a CPVT mouse model. Together, these studies suggest that CaMKII inhibition may be a translatable therapy for CPVT.

The CPVT engineered heart tissue model provided insights into mechanisms by which RYR2 mutation may cause arrhythmia at the tissue level. In isolated cell clusters, CPVT mutations are well established to cause aberrant Ca^{2+} release from the sarcoplasmic reticulum.[5,10–13] The resulting elevation of cytosolic Ca^{2+} drives depolarizing Na^{+}-Ca^{2+} exchange through the sodium-calcium exchanger, resulting in afterdepolarizations that can trigger subsequent action potentials.[1] In an ex vivo study of a mouse model of CPVT, triggered activity originating from His-Purkinje cells was reported to be the source of ventricular tachycardia, setting up reentry and mediating the transition from polymorphic ventricular tachycardia to ventricular fibrillation.[6] However, triggered activity in His-Purkinje cells alone is not sufficient for ventricular arrhythmia; a recent study demonstrated that the CPVT-inducing mutation must be present in working myocardium in addition to His-Purkinje to yield ventricular arrhythmia.[8] In the working myocardium, the 2 conditions necessary for focal activity to occur are unlikely to be fulfilled. First, the intracellular Ca^{2+} waves associated with increased diastolic Ca^{2+} and Ca^{2+} release events rarely cross cell borders[24]

and therefore do not form delayed afterdepolarizations synchronized among the quantity of cells necessary to elicit a triggered action potential. Second, electrotonic interaction between potential foci and neighboring tissue (so-called source-to-load mismatch) is expected, in contrast to observations made in single cells or clusters of few cells, to dampen the amount of local depolarization and to counteract or prevent ectopic action potential formation.[41] Likely for these same reasons, simulated exercise did not stimulate ectopic impulse initiation in our engineered CPVT tissues. Rather, a high pacing rate and isoproterenol stimulation markedly increased heterogeneity of Ca^{2+} wave propagation and elevated diastolic Ca^{2+} in CPVT tissue, resulting in local depolarization, propagation block, and subsequent reentry. This is consistent with computational modeling studies in which subthreshold afterdepolarizations increased repolarization heterogeneity and locally impaired cardiomyocyte excitability.[30] Thus, our data based on Ca^{2+} measurements, in combination with computational electric modeling and mouse models, suggest that CPVT mutations cause heterogeneity of action potential repolarization and excitability, creating a vulnerable substrate within working myocardium that sustains reentrant arrhythmias. Although reentrant spiral waves were initiated by collision of optically stimulated propagating Ca^{2+} transients with regions of focal conduction block in our experiments, triggered activity arising within the His-Purkinje system is likely to play a role as an initiating mechanism in whole hearts. Further studies in animal models are required to test this hypothesis.

Conclusions

We developed a human engineered tissue platform to elucidate the pathogenesis of inherited arrhythmias, and we used this platform to gain molecular and pathophysiological insights into CPVT. It is important to point out important limitations of the current engineered tissue model. One limitation is that we were unable to measure membrane voltage because currently available optical voltage sensors have greater toxicity, inadequate signal-to-noise properties, or spectral incompatibility with our optical actuator. Improved optical voltage sensors or refinements to the patterned substrate that permit multielectrode array recordings will allow measurement of membrane voltage or extracellular electrograms and provide additional insights into the mechanisms responsible for reentry. A second limitation is that current stem cell–derived cardiomyocytes are immature compared with adult cardiomyocytes,[42] and consequently, findings made in iPSC-CM systems such as dynamic conduction block precipitating reentry require further validation in animal models and in patients. Ongoing efforts to improve iPSC-CM maturity, combined with continued development of physiological, tissue-level arrhythmia assays, will further advance our ability to model human arrhythmias in in vitro systems. Finally, the molecular and cellular mechanisms that lead to heterogeneous calcium transient propagation and dynamic conduction block in CPVT opto-MTFs stimulated with isoproterenol and rapid pacing are unclear. Addressing these questions will be fertile ground for future studies.

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Acknowledgments

D.Z. and S-J.P. contributed equally to this study; their authorship order is interchangeable, and they are listed in alphabetical order. D.Z. and S-J.P. designed and performed experiments, analyzed data, organized figures, and wrote the paper. D.Z. developed the patient-derived and genome-edited iPSC-CMs and performed cell cluster assays, with help from Y.Q., P.Y., S.X., X.L., and F.L. S-J. P. developed the opto-MTF platform and analysis methods, performed experiments on opto-MTFs, and analyzed data. Y.L performed the Western blot experiments. J.G. and A.E.R. recruited the patient with CPVT. V.J.B. contributed to iPSC-CM characterization and with D.J.A. provided clinical data and insights. K.Y.L. conducted immunostaining. S.L.K. assisted with opto-MTF fabrication and analysis. K.Y.L. and F.S.P. optimized gelatin substrates and provided insights in experimental design and analysis. P.H.C. performed NRVM harvest for MTF optimization. A.G.K. provided mechanistic insights and edited the manuscript. W.T.P. conceived of and organized the project and cowrote the manuscript. K.K.P. supervised the project, developed the opto-MTF platform, and edited the manuscript.

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Disclosures

None.

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