Divergent Regulation of Ryanodine Receptor 2 Calcium Release Channels by Arrhythmogenic Human Calmodulin Missense Mutants

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Rationale: Calmodulin (CaM) mutations are associated with an autosomal dominant syndrome of ventricular arrhythmia and sudden death that can present with divergent clinical features of catecholaminergic polymorphic ventricular tachycardia (CPVT) or long QT syndrome (LQTS). CaM binds to and inhibits ryanodine receptor (RyR2) Ca release channels in the heart, but whether arrhythmogenic CaM mutants alter RyR2 function is not known.

Objective: To gain mechanistic insight into how human CaM mutations affect RyR2 Ca channels.

Methods and Results: We studied recombinant CaM mutants associated with CPVT (N54I and N98S) or LQTS (D96V, D130G, and F142L). As a group, all LQTS-associated CaM mutants (LQTS-CaMs) exhibited reduced Ca affinity, whereas CPVT-associated CaM mutants (CPVT-CaMs) had either normal or modestly lower Ca affinity. In permeabilized ventricular myocytes, CPVT-CaMs at a physiological intracellular concentration (100 nmol/L) promoted significantly higher spontaneous Ca wave and spark activity, a typical cellular phenotype of CPVT. Compared with wild-type CaM, CPVT-CaMs caused greater RyR2 single-channel open probability and showed enhanced binding affinity to RyR2. Even a 1:8 mixture of CPVT-CaM:wild-type-CaM activated Ca waves, demonstrating functional dominance. In contrast, LQTS-CaMs did not promote Ca waves and exhibited either normal regulation of RyR2 single channels (D96V) or lower RyR2-binding affinity (D130G and F142L). None of the CaM mutants altered Ca/CaM binding to CaM-kinase II.

Conclusions: A small proportion of CPVT-CaM is sufficient to evoke arrhythmogenic Ca disturbances, whereas LQTS-CaMs do not. Our findings explain the clinical presentation and autosomal dominant inheritance of CPVT-CaM mutations and suggest that RyR2 interactions are unlikely to explain arrhythmogenicity of LQTS-CaM mutations. (Circ Res. 2014;114:1114-1124.)

Key Words: calcium ■ long QT syndrome ■ catecholaminergic polymorphic ventricular tachycardia ■ ryanodine receptor calcium release channel ■ sarcoplasmic reticulum

Calmodulin (CaM) is an essential Ca-binding protein that transduces Ca signals in a wide range of biological processes including muscle contraction, inflammation, metabolism, memory, and immune responses. CaM functions as a Ca sensor for decoding Ca signals into downstream responses by undergoing conformational changes that promote binding to target proteins.1,2 CaM contains 4 EF-hand Ca-binding motifs located in 2 globular N-terminal (CaM-N) and C-terminal (CaM-C) domains connected by a flexible linker.3,4 Humans have 3 CaM genes—CALM1, CALM2, CALM3—encoding the identical amino acid sequence, which is perfectly conserved among vertebrates and highly conserved in nonvertebrates. CaM gene deletion is lethal in yeast5 and Drosophila.6

In This Issue, see p 1069

Recent genetic studies have identified CaM missense mutations in humans with severe ventricular arrhythmia and sudden cardiac death susceptibility,7,8 albeit with distinct clinical presentations: 2 mutations in CALM17 were associated with stress-induced polymorphic ventricular tachycardia reminiscent of

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catecholaminergic polymorphic ventricular tachycardia (CPVT-CaMs), whereas 3 other mutations in either CALM1 or \textit{CALM} \textsubscript{2} \textsuperscript{8} led to recurrent cardiac arrest in infancy associated with severe QT prolongation reminiscent of a long QT syndrome (LQTS-CaMs). A recent report has demonstrated that all 3 LQTS-CaMs (D96V, D130G, and F142L) suppress Ca-dependent inactivation of L-type Ca currents and cause action potential prolongation, which can explain the long QT phenotype of mutation carriers. \textsuperscript{9} In contrast to LQTS, the cardiac action potential and hence the QT interval are not altered in CPVT, \textsuperscript{10} and the mechanism underlying CPVT caused by CaM mutations is not known.

CPVT can be caused by mutations in genes involved in Ca release from sarcoplasmic reticulum (SR) during excitation–contraction coupling. \textsuperscript{11} The most common autosomal dominant form of CPVT is associated with mutations in the \textit{RYR2} gene encoding the cardiac ryanodine receptor (RyR2) SR Ca release channel. \textsuperscript{11} Autosomal recessive CPVT is less common and has been associated with mutations in the genes encoding RyR2-binding proteins calsequestrin-2 (Casq2) and triadin. \textsuperscript{11} Disease-causing CPVT mutations render RyR2 Ca release channels prone to spontaneous opening, resulting in spontaneous Ca release and propagated Ca waves that trigger membrane depolarizations, premature beats, and polymorphic ventricular tachycardia during exercise or emotional stress. \textsuperscript{12}

RyR2 channels bind CaM in cardiac muscle, \textsuperscript{13} and this interaction reduces channel open probability. \textsuperscript{14} Previous work has suggested that some CPVT-linked \textit{RYR2} mutations can impair CaM binding to RyR2 and thereby increase RyR2 channel openings and spontaneous Ca release. \textsuperscript{15} Although impaired binding to RyR2 by mutant CPVT-CaMs is possible, all 3 CaM genes are expressed in the human heart, \textsuperscript{8} and it is not clear how a single-point mutation in 1 of 6 CaM alleles could influence RyR2 activity to produce CPVT, especially if binding of the mutant CaM to its target on RyR2 is reduced. Alternatively, impaired Ca binding to the C-lobe of CaM has recently been shown to promote spontaneous Ca release in human embryonic kidney cells expressing RyR2 channels. \textsuperscript{16} Although disease-linked CaM mutants were not investigated in that study, \textsuperscript{16} all LQTS-CaMs disrupt C-lobe Ca binding to CaM. \textsuperscript{8} This result would predict that all LQTS-CaMs would also cause RyR2 dysregulation, which may contribute to the severe arrhythmia phenotype of patients carrying LQTS-linked CaM mutations. Hence, in addition to CPVT-CaMs, we also investigated the effect of LQTS-CaMs on RyR2 channels and myocyte Ca handling and thereby gain mechanistic insight into how CaM mutations cause ventricular arrhythmias and sudden death in humans.

### Nonstandard Abbreviations and Acronyms

| CaM      | calmodulin |
| CaMKII   | Ca/CaM kinase II |
| Casq2    | calsequestrin-2 |
| CPVT     | catecholaminergic polymorphic ventricular tachycardia |
| LQTS     | long QT syndrome |
| P\textsubscript{o} | single-channel open probability |
| RyR2     | ryanodine receptor 2 |
| SR       | sarcoplasmic reticulum |

## Animal Use

The use of animals in this study was approved by the Institutional Animal Care and Use Committees and performed in accordance with National Institutes of Health guidelines.

## Measurement of Ca Binding to CaM

To study the consequences of the human CaM disease-associated mutations on Ca binding, we bacterially expressed and purified recombinant wild-type (WT) and mutant CaMs. Ca-binding affinities for WT, N54I, and N98S-CaM proteins were determined as described. \textsuperscript{8} Briefly, macroscopic-binding constants for the pairs of Ca-binding sites in CaM-N and CaM-C were measured by monitoring the intrinsic tyrosine and phenylalanine fluorescence of the protein during the course of a Ca titration. \textsuperscript{12} The data were analyzed by plotting the normalized fluorescence signal versus free [Ca] and fitting to the model independent 2-site Adair function. The dissociation constants (K\textsubscript{d}) for each domain are reported as the average value for the pair of sites by taking the square root of K2 from the Adair equation.

### Ca Wave Experiments in Ventricular Myocytes

Single ventricular myocytes from 12- to 16-week-old C57BL/6 mice were isolated as described. \textsuperscript{16} Myocytes were permeabilized with saponin (40 μg/mL) for 60 seconds and placed in internal solution composed (in mmol/L) of K-aspartate 100, KCl 15, KH\textsubscript{2}PO\textsubscript{4} 5, MgCl\textsubscript{2} 0.75, dextran (40000) 4%, HEPES 10, MgATP 5, phosphocreatine di-Na 10, creatine phosphokinase 10 U/mL, glutathione (reduced) 10, and Fluor 0.025. Free [Ca]\textsubscript{0} was 120 mmol/L. To allow equilibration of CaM binding to cellular targets, all Ca wave measurements were taken after 30-minute incubation with either WT or mutant CaM alone, or with a mixture of WT and mutant CaMs. Free [CaM] was kept at the physiological concentration of 100 nmol/L. \textsuperscript{16} Ca waves in myocytes were imaged with an confocal microscope in line scan mode. Ca wave analysis was performed as described. \textsuperscript{20} Given the variability between different experimental days, the Ca wave frequency and Ca amplitudes data were normalized to the mean of WT-CaM group obtained on the same day.

### CaM Binding to RyR2

To resolve CaM binding to RyR2, we used a fluorescence resonance energy transfer (FRET)–based competition binding assay that detects acceptor-labeled CaM binding in the proximity of donor-labeled FKBP pretargeted to RyR2. \textsuperscript{21} Briefly, SR vesicles from porcine ventricular myocardium decorated with donor-labeled FKBP were used to prepare the FRET samples consisting of 3 mg/mL SR, WT or mutant CaM, 100 nmol/L acceptor-labeled CaM, 20 mmol/L K-PIPES (pH 7.0), 150 mmol/L KCl, 5 mmol/L glutathione, 1 mmol/L EGTA, 0.1 mg/mL BSA, 1 μg/mL aprotinin/leupeptin, and sufficient CaCl\textsubscript{2} to yield the indicated free [Ca]\textsubscript{0}. \textsuperscript{22} After a 2.5-hour incubation at 25°C, the samples were transferred to a 384-well plate and fluorescence spectra read in a fluorescence plate reader. FRET was calculated based on the fractional decrease of donor fluorescence (F\text{donor}) in the presence of acceptor (F\text{acceptor}), according to FRET=1–F\text{donor}/F\text{acceptor}.

### Table. Fold Reduction in Ca-Binding Affinity of LQTS- and CPVT-CaMs

<table>
<thead>
<tr>
<th>CaM Mutation</th>
<th>CaM-C Domain K\textsuperscript{d} (Fold Reduction)</th>
<th>CaM-N Domain K\textsuperscript{d} (Fold Reduction)</th>
<th>Clinical Arrhythmia Syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>D130G</td>
<td>53.6 (No change)</td>
<td>N/A</td>
<td>LQTS</td>
</tr>
<tr>
<td>D96V</td>
<td>13.6 (No change)</td>
<td>N/A</td>
<td>LQTS</td>
</tr>
<tr>
<td>F142L</td>
<td>5.4 (No change)</td>
<td>N/A</td>
<td>LQTS</td>
</tr>
<tr>
<td>N98S</td>
<td>3.3 (No change)</td>
<td>N/A</td>
<td>CPVT</td>
</tr>
<tr>
<td>N54I</td>
<td>No change</td>
<td>No change</td>
<td>CPVT</td>
</tr>
</tbody>
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K\textsuperscript{d} values were obtained from Figure 1 and reference B and compared with control K\textsuperscript{d} values for WT-CaM. CaM indicates calmodulin; CPVT, catecholaminergic polymorphic ventricular tachycardia; and LQTS, long QT syndrome.
Single-Channel Recording of RyR2 Activity

RyR2 was isolated from sheep hearts and incorporated into artificial lipid bilayers as described.\textsuperscript{22} RyR2 gating was measured at −40 mV in cytoplasmic solutions containing 250 mmol/L Cs, 2 mmol/L ATP, 10 μmol/L KN93, and Ca of either 0.1 or 1 μmol/L. Luminal solutions contained 250 mmol/L Cs and 0.1 mmol/L Ca (pH 7.4). A local perfusion technique that allows rapid fluid exchange (<5 seconds)\textsuperscript{23} was used to apply vehicle solutions to RyR2 or vehicle plus 100 nmol/L WT-CaM or CaM mutants. Single-channel recordings were analyzed for open probability using a 50% threshold method.

CaM-Kinase Activation

WT and mutant CaM-binding affinity for Ca/CaM kinase II (CaMKII) was measured using a plasmid encoding for FRET-based Camui as previously described.\textsuperscript{24} A more detailed description of the experimental methods can be found in the online-only Data Supplement.

Figure 1. Ca-binding affinity of catecholaminergic polymorphic ventricular tachycardia (CPVT)-calmodulins (CaMs). Ca\textsuperscript{2+} titration curves for wild-type (WT) and CPVT-CaMs (N54I and N98S) for the CaM-C and CaM-N domain. Data were used to derive dissociation constants (K\textsubscript{d}, in μmol/L) for each domain. C domain: 3.3±0.2 (WT), 3.1±0.2 (N54I), and 1±1 (N98S). N domain: 23±3 (WT), 23±3 (N54I), and 22±2 (N98S). Values are averages of 3 experiments, and error was determined by analysis of the curve fits.

![Figure 1](http://circres.ahajournals.org/)

Figure 2. Only catecholaminergic polymorphic ventricular tachycardia (CPVT)-calmodulins (CaMs) promote spontaneous Ca\textsuperscript{2+} waves in permeabilized myocytes. A, Representative line scans (red arrow) from permeabilized mouse ventricular myocytes after 30-minute incubation with either wild-type (WT) or mutant CaMs (100 nmol/L). CPVT-CaMs promoted higher Ca wave frequency (B) and lower Ca wave amplitude (C). After permeabilization, myocytes were incubated in internal solution composed of 120 nmol/L free [Ca\textsuperscript{2+}], 100 μmol/L EGTA, and 25 μmol/L Fluo 4. Bars represent mean±SE of values normalized by WT values on each experimental day. WT (white, n=45), D96V (light gray, n=33), D130G (gray, n=13), F142L (dark gray, n=20), N54I (red, n=35), and N98S (blue, n=35). Casq2KO: myocytes isolated from Casq2 null mice and incubated with WT-CaM (violet, n=21; *P<0.05, **P<0.01 vs WT-CaM).

![Figure 2](http://circres.ahajournals.org/)
Results

Divergent Effects of CaM Mutations on C-Domain Ca-Binding Affinity

CaM has 2 high-affinity Ca-binding sites (EF-hand III and IV) in the C domain and 2 slightly lower affinity Ca-binding sites (EF-hand I and II) in the N domain. The N54I mutation, which is located 2 residues before the first Ca-coordinating residue of EF-hand II, had no significant effect on CaM-C or CaM-N Ca-binding affinity compared with WT-CaM (Figure 1). In contrast, the N98S mutation, which is in EF-hand III of CaM-C, decreased Ca affinity in CaM-C but had no effect on CaM-N (Figure 1). Our results are in agreement with a previous report that investigated only the C-domain Ca-binding properties of these 2 mutants. The Table compares the Ca-binding affinity of CPVT-CaMs with those reported for the 3 LQTS-CaMs. Note that all LQTS mutations affect CaM-C Ca affinity to a greater extent than the CPVT mutations, which either have no or only modest effects on Ca binding. These data suggest that lower Ca-binding affinity is not the critical mechanism of CPVT caused by CaM mutants. Conversely, there is good correlation between impaired Ca binding and the LQTS phenotype.

Only CPVT-CaM Mutants Promote Ca Waves

We next examined the functional consequences of CPVT-CaMs and LQTS-CaMs on cellular Ca handling in saponin-permeabilized murine ventricular cardiomyocytes. Experiments were performed at physiological free [Ca] of 100 nmol/L and diastolic free [Ca] of 120 nmol/L. Under those conditions, myocytes exhibit spontaneous Ca waves (Figure 2A). In this bioassay, CPVT mutations in RyR2 or Casq2 that promote higher RyR2 channel open probability cause greater frequency and lower amplitude of spontaneous Ca waves. Furthermore, drugs such as flecainide that inhibit RyR2 channels and reduce Ca wave frequency in this bioassay have been shown to prevent CPVT in mice and humans. LQTS-CaMs either had no effect or evoked slightly lower Ca wave frequency (Figure 2). In contrast, both CPVT-CaMs caused significantly greater Ca wave frequency similar to the higher Ca wave frequency observed in WT-CaM–treated myocytes lacking Casq2 (Casq2 KO, Figure 2), an established mouse model of genetic CPVT. Moreover, flecainide treatment effectively suppressed Ca waves (Online Figure I), suggesting that flecainide may be useful for treating CPVT associated with CaM mutations.

One plausible explanation for their differential effect on Ca waves is that CPVT- and LQTS-CaMs bind to RyR2 with different affinity. Hence, we used a FRET-based assay to specifically measure CaM bound to RyR2 in SR vesicles (as opposed to total CaM bound to SR), RyR2-specific CaM binding was measured by FRET between fluorescent FKBP12.6 and fluorescent CaM. Figure 3A illustrates how WT and CaM mutants compete with 100 nmol/L fluorescent CaM and reduce FRET, at either 30 nmol/L or 30 μmol/L [Ca]. Strikingly, both CPVT-CaMs displaced fluorescent CaM more effectively than did WT-CaM (P<0.05; n=4) at low [Ca] relevant to diastolic [Ca] in the heart. At high [Ca] (30 μmol/L), which should saturate CaM, CPVT-CaMs were no better than WT-CaM at displacing fluorescent CaM (Figure 3).

Figure 3. Catecholaminergic polymorphic ventricular tachycardia (CPVT)-calmodulins (CaMs) bind with higher affinity to ryanodine receptor 2 (RyR2) Ca release channels in nanomolar Ca conditions. A. In cardiac sarcoplasmic reticulum vesicles stripped of native CaM, RyR2 was prelabeled with AF488-FKBP (donor) and then incubated with 100 nmol/L AF568-CaM (acceptor) and a series of concentrations of wild-type (WT) and mutant (Mut) CaMs (D96V, light gray; D130G, gray; F142L, dark gray; N54I, red; and N98S, blue). AF568-CaM binding to RyR2 was determined from the decrease in AF488-FKBP fluorescence because of fluorescence resonance energy transfer. Data are means±SE (n=3–4) for each concentration. B, Mean ratio of mutant and WT IC50 of CaM binding to RyR2 (*P<0.05 vs WT-CaM, n=3–4 experiments per group).

Only CPVT-CaM Mutants Increase RyR2 Single-Channel Open Probability

To test this hypothesis directly, we next applied 100 nmol/L of WT-CaM, CPVT-CaM (N54S and N98S) or LQTS-CaM (D96V) to single RyR2 channels incorporated into lipid bilayers. Experiments were performed at cytosolic [Ca] of 1 μmol/L to activate RyR2 channels. All solutions contained KN93 (10 μmol/L) to prevent CaMKII activation. As illustrated in Figure 4A, adding WT-CaM to CaM-free RyR2 channels significantly reduced their activity (ie, lowered
single-channel open probability [P_o]), which is consistent with the inhibitory action of CaM on RyR2 channels.30 In contrast, single-channel P_o was unchanged after application of either CPVT-CaM (N54I and N98S), resulting in a significantly higher RyR2 single-channel P_o compared with WT-CaM (Figure 4A and 4C). However, the effect of LQTS-CaM D96V on single RyR2 channels was comparable to that of WT-CaM (Figure 4A and 4C), a result that is consistent with the lack of Ca wave activation by D96V (Figure 2). We next performed experiments at cytosolic [Ca] of 0.1 μmol/L to mimic diastolic conditions. Applying CPVT-CaM N54I to RyR2 channels devoid of endogenous CaM significantly increased RyR2 channel activity (Figure 4B and 4D), whereas N98S-CaM had no significant effects on RyR2 channel activity (Figure 4D). As a result, N54I-CaM significantly increased RyR2 channel P_o compared with either WT-CaM or N98S-CaM (Figure 4D). These results indicate that the N98S CPVT mutant fails to inhibit RyR2 channels, whereas N54I not only lacks inhibitory action but also directly activates RyR2 channels at diastolic [Ca].

We next measured the effect of CPVT-CaMs on Ca sparks, which are Ca release events by clusters of RyR2 channels in myocytes.31 To prevent wave propagation, Ca sparks were measured at 50 nmol/L free [Ca] with strong buffering by 0.5 mmol/L EGTA. SR Ca content was assessed by rapid caffeine application because it strongly influences SR Ca leak,32 Ca spark frequency, and amplitude.33 Adding 100 nmol/L WT-CaM to myocytes predepleted of endogenous CaM evoked lower Ca spark frequency (Figure 5A and 5B) and lower SR Ca leak, which also resulted in greater SR Ca content (Figure 5C and 5D). The rise in SR Ca content by itself would tend to promote Ca sparks, so the lower Ca spark frequency underestimates the RyR2 effect of CaM. Compared with WT-CaM, both CPVT-CaMs evoked significantly greater Ca spark frequency (Figure 5A and 5B), and this effect was enhanced by cAMP (Online Figure II), which was used to

Figure 4. Abnormal regulation of single ryanodine receptor 2 (RyR2) channels by catecholaminergic polymorphic ventricular tachycardia (CPVT)–associated calmodulins (CaM) mutants. A, Continuous records of RyR2 gating in the absence of CaM. Cytoplasmic bath contained 1 μmol/L [Ca]. Arrows indicate addition of 100 nmol/L of wild-type (WT) CaM, CPVT-CaMs (N54I and N98S), or long QT syndrome (LQTS)-CaM (D96V). Solution changes were achieved within <5 seconds using local perfusion of the RyR2 (see Methods). Average open probability (P_o) values before and after adding CaM are given at the left and right of each trace, respectively. B, Continuous record of a single RyR2 beginning in the absence of CaM at 0.1 μmol/L [Ca]. Addition of 100 nmol/L N54I-CaM (arrow) increased RyR2 channel activity. Average effect of WT and mutant CaMs on RyR2 P_o at (C) 1 μmol/L and (D) 0.1 μmol/L cytosolic [Ca]. P_o values were obtained from 1 to 2 minutes of continuous recording under each condition. Before averaging, each P_o value was normalized to the P_o of the same RyR2 channel obtained before adding CaM. *P<0.05, **P<0.01, ***P<0.001 by Mann–Whitney test, #P<0.05, ##P<0.01 by Wilcoxon signed-rank test vs CaM-free RyR2 (n=10 to 14 records per group).
model adrenergic stress. Despite our conditions, which robustly suppress Ca waves in WT myocytes, Ca waves were observed occasionally with CPVT-CaMs (especially N98S) but not in either CaM-free or WT-CaM exposure. This agrees with the promotion of Ca waves by N54I and N98S illustrated in Figure 2 (at higher [Ca] and lower [EGTA]). CPVT-CaMs also reduced spark amplitude and increased spark duration and spark width (Online Table I). These data are consistent with the single-channel results (Figure 4) and indicate that CPVT-CaMs promote rather than inhibit RyR2 channel activity, resulting in an increased SR Ca leak and reduced SR Ca content.

**CPVT-CaMs Exhibit Dominant-Activating Effects on Ca Waves**

Given that only 1 out of 6 CaM alleles in patients has a mutation, another important question is whether CPVT-CaMs can exert a dominant effect on RyR2 function in the presence of excess WT-CaM. To address this question, we performed mixing studies that tested the effects of various ratios of CPVT-CaMs and WT-CaM on Ca wave frequency (Figure 6). We found that even in the presence of 8-fold excess of WT-CaM (87.5%), CPVT-CaMs promoted significantly higher Ca wave frequencies (Figure 6B). These results are consistent with the higher affinity of CPVT-CaMs for RyR2 at low Ca (Figure 3), which suggests that the fraction of RyR2 bound to N54I or N98S (versus WT-CaM) would be higher than their relative expression level.

**CaMKII Does Not Contribute to RyR2 Activation by CPVT-CaMs**

An important CaM target in cardiac muscle is CaMKII. CaMKII activation has been implicated in several heart diseases associated with increased SR Ca leak and Ca-triggered arrhythmias including CPVT.34 RyR2 phosphorylation by CaMKII increases RyR2 P_o and the frequency of spontaneous Ca waves.35,36 Hence, we tested whether CaMKII activation by disease-associated CaM mutants contributes to their effect on Ca waves. Blocking CaMKII activation by disease-associated CaM mutants contributes to their effect on Ca waves. Blocking CaMKII activation by KN93 significantly reduced Ca wave frequency in myocytes incubated with WT-CaM or LQTS-CaM D96V but had no effect on Ca wave frequency in myocytes exposed to the other CaM mutants (Figure 7A and 7B). The results raise the possibility that (other than D96V) CaM mutants might impede CaMKII activation by Ca/CaM. To test this idea more directly, we measured the [CaM] dependence of CaMKII activation using Camui, a FRET-based reporter of CaMKII activation state.24 Camui FRET is reduced when Ca/CaM binds to and opens up the CaMKII structure, a critical step in activation. None of the CaM mutants significantly altered the apparent CaMKII

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**Figure 5. Catecholaminergic polymorphic ventricular tachycardia (CPVT)-calmodulins (CaMs) promote Ca sparks and lower sarcoplasmic reticulum (SR) Ca content.**

A, Representative line-scan images of Ca sparks in permeabilized rat ventricular myocytes in CaM-free, wild-type (WT) CaM, and CPVT-CaMs (N54I and N98S). Line plots of spark events (red arrow) are indicated below their images. B, Average Ca spark frequency. Bars represent mean+SE. CaM-free (black, n=5), WT (white, n=9), N54I (red, n=8), N98S (blue, n=10). C, Line scan (top) and line plot (bottom) examples of SR Ca^2+ content evaluated by 10 mmol/L caffeine-evoked Ca transient in WT-CaM. D, Average SR Ca content. CaM-free (black, n=5), WT (white, n=6), N54I (red, n=6), N98S (blue, n=7). [Ca]=50 nmol/L, EGTA=0.5 mmol/L, WT or mutant CaMs=100 nmol/L (**P<0.01, ***P<0.001 vs WT-CaM).
affinity at 200 μmol/L [Ca] (Figure 7C and 7D). However, WT and D96V had the lowest K_d values, which would be consistent with their having some CaMKII-dependent effect on Ca waves (Figure 7A). Taken together, these results essentially exclude CaMKII activation as a mechanism responsible for activation of RyR2 and arrhythmogenic Ca waves generated by CPVT-CaMs.

**Discussion**

The major finding reported here is that unlike WT-CaM, CPVT-CaMs exert an activating effect on RyR2 Ca release channels and evoke higher frequencies of spontaneous Ca waves in murine ventricular myocytes. The cellular Ca-handling defect induced by CPVT-CaMs is similar to that reported for CPVT-linked mutations in RYR2 and CASQ2. CPVT-CaMs bind to RyR2 with higher affinity than WT-CaM, which likely contributes to their dominant action in the heart. At the same time, CPVT-CaMs have near normal Ca-binding affinities and may function normally as Ca sensors, which could explain why 2 mutations in the ubiquitously expressed CALM1 gene are only associated with a specific CPVT phenotype. In contrast, LQTS-CaMs lack direct effects on SR Ca release, which can be explained either by their reduced RyR2-binding affinity (D130G and F142L) or normal inhibitory regulation of RyR2 channels (D96V). Collectively, our results provide new mechanistic insight into the divergent human arrhythmia phenotypes caused by CaM mutations.

Studying mouse models expressing human CPVT-linked mutations, investigators have consistently found higher rates of spontaneous Ca release, delayed afterdepolarizations, and triggered beats in cardiac muscle. Furthermore, drugs that suppress the spontaneous SR Ca release in single cells are also effective in preventing exercise or stress-induced CPVT in mice and humans. Hence, it is generally accepted that the dysfunctional and premature spontaneous Ca release from the SR is the culprit of the CPVT observed in vivo. Investigators have proposed several different mechanisms to explain the propensity of spontaneous SR Ca release associated with CPVT mutations: loss of regulation of RyR2 mutant channels by luminal Ca resulting in store-overload–induced Ca release, defective interdomain interaction of RyR2 mutant channels, reduced FKBP12.6 binding to RyR2, loss of luminal Ca sensing or Ca release refractoriness because of loss of Casq2 or triadin, or impaired CaM binding to protein kinase A-phosphorylated RyR2.
mutant channels.\textsuperscript{15} Our results suggest a new mechanism that is responsible for CPVT caused by \textit{CALM1} mutations, a mechanism that is distinct from those reported previously for CPVT mutations in other genes: CPVT-CaMs bind with greater affinity to RyR2 channels but at the same time fail to inhibit or even activate RyR2 channels, rendering them hyperactive, which generates arrhythmogenic Ca waves and leads to SR Ca store depletion. However, the CPVT-CaMs produced this Ca wave activation by distinct RyR2 single-channel phenotypes. The N-domain mutation N54I seems to activate RyR2 channels directly in an agonist-like action similar to caffeine. In contrast, N98S located in the C domain does not activate RyR2 channels by itself but fails to inhibit RyR2 channels, especially at higher cytosolic [Ca]. These different single-channel mechanisms of action could explain why the N54I mutant had more potent activating effects on Ca waves and Ca sparks at low [Ca] than N98S (Figures 2 and 5).

Both CPVT-CaMs exhibit dominant-activating effects on Ca waves when mixed with WT-CaM (Figure 6), which is consistent with an autosomal dominant inheritance pattern in humans. What are possible reasons for this functional dominance? One possibility is based on the fact that the tetrameric RyR2 Ca release channel complex has 4 CaM-binding sites.\textsuperscript{44} Binding of only a single mutant CaM may be sufficient to disrupt the CaM-dependent regulation of the RyR2 channel complex, analogous to the dominant effects of point mutations in K-channel monomers that function as tetrameric complexes in the cell membrane. Thus, single aminoacid changes in CaM may substantially alter the gating of a large RyR macromolecular complex. For example, WT-CaM has an activating effect on skeletal RyR1 channels at nanomolar concentrations.\textsuperscript{4}

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**Figure 7.** Ca/calmodulin kinase II (CaMKII) activation is not responsible for the effect of catecholaminergic polymorphic ventricular tachycardia (CPVT)-calmodulins (CaMs) on Ca waves. A and B, Effect of CaMKII inhibition with KN93 on Ca wave frequency and amplitude. Permeabilized myocytes were incubated with 100 nmol/L of CaM mutants in presence or absence of KN93 (1 \( \mu \)mol/L, 30-minute preincubation). Bars represent mean±SE. Wild type (WT; white, n=40), D96V (light gray, n=20), D130G (gray, n=15), F142L (dark gray, n=20), N54I (red, n=29), N98S (blue, n=12), \( *P<0.05 \) vs+KN93. With the exception of WT-CaM and long QT syndrome (LQTS)-CaM D96V, CaMKII activation by CaM did not contribute to the effect of CaM mutants on Ca waves. C, CaM-dependent activation of CaMKII (WT-CamuI) measured in human embryonic kidney (HEK) 293 cell lysate at 37°C with saturating Ca (200 \( \mu \)mol/L). CaM±1 mmol/L EGTA (black, n=13 each), N54I (red, n=9), N98S (blue, n=10), D96V (light gray, n=11), F142L (gray, n=9), and D130G (dark gray, n=10). Decreases in fluorescence resonance energy transfer (FRET) were normalized for each mutant (maximal FRET change was similar to WT for all mutants, except F142L and D96V where it was smaller). D, [CaM] for half-maximal change in FRET (K_{0.5}) obtained from C for WT and mutant CaM.
[Ca] but an inhibitory effect at high micromolar [Ca],45 suggesting that the CaM-binding domain in both RyR1 and RyR2 can transduce large changes in gating that can be either stimulatory or inhibitory. It is possible that the CPVT-CaMs exert an activating effect on RyR2 gating at diastolic [Ca] (analogous to effects of WT-CaM on RyR1), despite having only slightly altered Ca affinity (Table).46 In their initial report of CaM mutations associated with a clinical CPVT phenotype, Nyegaard et al7 assessed the interaction of the 2 CPVT-CaMs with a RyR2-derived peptide encompassing the putative CaM-binding domain by monitoring the RyR2 peptide’s Trp3586 fluorescence emission spectra. Although results for N54I were not different from WT-CaM, the interaction of N98S-CaM with the RyR2 peptide was impaired, selective at 100 nmol/L [Ca] (but not at 1–200 μmol/L [Ca]). CaM binding to native, full-length RyR2 channels or RyR2 function was not assessed in that report. Based on their results, Nyegaard et al7 concluded that the loss of function with regard to the RyR2 interaction may lead to inappropriate RyR2 leakiness, similar to CPVT caused by RyR2 mutations that reduce CaM binding to RyR2.15 In contrast, our studies using native RyR2 channels suggest a mechanism of action that is different from that proposed for RyR2 mutations: CaM mutations that reduce binding affinity to RyR2 (ie, LQTS-CaM D130G and F141L; Figure 3) do not cause CPVT. Rather, we found that CaM mutations resulting in CPVT (CPVT-CaM N54I and N98S) bind to native RyR2 with higher affinity than WT-CaM (Figure 3) and that this interaction changes the CaM effect on RyR2 from an inhibitory to a stimulatory effect, especially for N54I. Given that there are 3 genes encoding identical CaM proteins which are all expressed in the human heart,8 it is likely that both mechanisms (increased RyR2 binding and stimulatory action) contribute to the dominant effect of the CALM1 mutations observed in patients with CPVT. Even a minority of RyR2 channels with mutant CaM bound could be enough to nucleate Ca-induced Ca release as spontaneous Ca waves and trigger CPVT in vivo.

CaM binds to multiple other targets in the heart; hence, we cannot exclude that other mechanisms contribute to the autosomal dominant inheritance of CPVT caused by CALM1 mutations. One important target is CaMKII, which after activation by Ca/CaM can phosphorylate RyR2 channels, produce spontaneous Ca waves, deplete SR Ca stores and trigger arrhythmia.39 However, based on our findings that CaM mutants did not differentially regulate CaMKII (Figure 7) and CaMKII inhibition by KN93 did not prevent the effects of CPVT-CaMs on Ca waves (Figure 7) and RyR2 single channels (Figure 4), altered CaMKII regulation is probably not a culprit in CPVT caused by CaM mutations. Another important CaM target involved in arrhythmogenesis is the L-type Ca channel Ca,1,2. Ca-free-CaM prebound the C terminus of Ca,1,2 importantly regulates Ca-dependent channel inactivation.47 CaM mutations in EF-hand motifs that prevent Ca binding to CaM severely disrupt Ca-dependent inactivation of Cav1.2 channels,48 an effect that has been recently reported for the 3 LQTS-CaMs (D96V, D130G, and N142L).9 Accordingly, impaired Ca-dependent inactivation and the ensuing excess L-type Ca current during the phase III of the action potential has been proposed as the underlying mechanism responsible for the QT prolongation caused by LQTS-CaMs,9 all of which are located in an EF-hand and significantly impair Ca binding to CaM (Table). In contrast, CPVT-CaMs exhibit only modest or no impairment of Ca binding (Table), a likely explanation as to why these mutations do not cause QT prolongation. Other potentially arrhythmogenic CaM targets in the heart include the cardiac Na channels, K channels, and calcineurin.2,28 It may be worthwhile to explore how mutant CaMs interact with and regulate those targets as well.

An important result of our studies is that LQTS-CaMs had no effect on SR Ca release (Figure 2), which is consistent with the lack of a clinical CPVT phenotype in this group of mutation carriers.8 For the LQTS-CaM D96V, which has normal RyR2-binding affinity (Figure 3), we show that it is capable of physiological inhibition of single RyR2 channels akin to WT-CaM (Figure 4). For the other 2 LQTS-CaMs (D130G and F142L), the absence of Ca waves modulation may be explained by their low-binding affinity to RyR2 compared with WT-CaM (Figure 3). Because all LQTS-CaMs also drastically impair C-lobe Ca binding (Table), our results seem at odds with a recent report suggesting that impaired Ca binding to the C-lobe of CaM promotes spontaneous Ca release.16 A possible explanation for the differences is that those experiments were conducted in HEK cells over-expressing mutant CaMs and LQTS-CaM mutations were not studied,16 whereas our experiments were performed at physiological free [CaM] of 100 nmol/L in native ventricular myocytes. Although we did not study the effect of CaM mutants on arrhythmias at the cellular or in vivo level, we nevertheless find that the divergent actions of the mutant CaMs on Ca binding, Ca wave modulation, and RyR2 regulation were consistent with the distinct human arrhythmia phenotypes. Thus, the in vitro assays described in our report could be useful for evaluating the pathogenicity of other human CaM mutations that undoubtedly will be discovered in the future.

**Acknowledgments**

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**Disclosures**

None.

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Novelty and Significance

CaM missense mutations are associated with arrhythmia and sudden cardiac death susceptibility in humans, but how mutant CaMs cause arrhythmia susceptibility is not known. Because RyR2 channels are regulated by CaM and abnormal RyR2 channel activity can trigger ventricular arrhythmias, we hypothesized that arrhythmogenic CaM mutants alter RyR2 function. We find that unlike wild-type CaM, CPVT-CaMs activate RyR2 and promote spontaneous Ca release in myocytes. The cellular Ca-handling defect induced by CPVT-CaMs is similar to that reported for CPVT-linked mutations in RYR2 and CASQ2 genes. CPVT-CaMs bind to RyR2 with higher affinity than wild-type CaM, which likely contributes to their dominant action in the heart. At the same time, CPVT-CaMs have near normal Ca-binding affinities and may function normally as Ca sensors, which could explain why mutations in the ubiquitously expressed CaM are only associated with a specific CPVT phenotype. In contrast, LQTS-CaMs lack direct effects on Ca release because of either their reduced RyR2-binding affinity or normal inhibitory regulation of RyR2 channels. Collectively, our results provide new mechanistic insight into the divergent human arrhythmia phenotypes caused by CaM mutations. Furthermore, the in vitro assays described here could be useful for evaluating the pathogenicity of other human CaM mutations that undoubtedly will be discovered in the future.
Divergent Regulation of Ryanodine Receptor 2 Calcium Release Channels by Arrhythmogenic Human Calmodulin Missense Mutants

Hyun Seok Hwang, Florentin R. Nitou, Yi Yang, Kafa Walweel, Laetitia Pereira, Christopher N. Johnson, Michela Faggioni, Walter J. Chazin, Derek Laver, Alfred L. George, Jr, Razvan L. Cornea, Donald M. Bers and Björn C. Knollmann

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Flecainide reduced spontaneous Ca wave frequency in permeabilized myocytes. (A) Representative line scans from permeabilized ventricular myocytes before (-) and 5 min after (+) flecainide (25 μM) application after 30 min incubation with either WT or CPVT-CaMs N54I, N98S (100nM). Exposure to vehicle (DMSO) had no significant effect on Ca wave frequency (data not shown). (B) Average data. Bars are mean±SEM. WT (white, n=5), N54I (red, n=10), N98S (blue, n=10). **P<0.01 vs. vehicle.
Online Figure II. cAMP (10µM) increase Ca spark frequency both for WT and mutant CaMs. WT (white, n=5), N54I (red, n=5), N98S (blue, n=5). [Ca]ᵢ = 30 nM, EGTA = 0.5 mM, [WT] or [mutant CaMs] = 100 nM. *P<0.05, ***P<0.001, control vs cAMP.
Online Table I. Effect of CPVT-CaMs on Ca spark properties

<table>
<thead>
<tr>
<th></th>
<th>FDHM (ms)</th>
<th>Amplitude (ΔF/F0)</th>
<th>FWHM (µm)</th>
<th>No. of sparks</th>
</tr>
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<tbody>
<tr>
<td>CaM-free</td>
<td>48.7 ± 0.37*</td>
<td>0.61 ± 0.01*</td>
<td>2.01 ± 0.02*</td>
<td>2182</td>
</tr>
<tr>
<td>WT</td>
<td>42.2 ± 0.24</td>
<td>0.84 ± 0.01</td>
<td>1.83 ± 0.01</td>
<td>1738</td>
</tr>
<tr>
<td>N54I</td>
<td>50.0 ± 0.29*</td>
<td>0.59 ± 0.01*</td>
<td>1.96 ± 0.01*</td>
<td>1736</td>
</tr>
<tr>
<td>N98S</td>
<td>49.3 ± 0.37*</td>
<td>0.51 ± 0.01*</td>
<td>1.98 ± 0.01*</td>
<td>2691</td>
</tr>
</tbody>
</table>

FDHM: full duration at half maximum, FWHM: full width at half maximum. *P<0.001, vs WT.
Online Methods Supplement

Animal use. The use of animals in this study was approved by the Animal Care and Use Committees of Vanderbilt University, Nashville, TN, USA, University of California Davis, CA, USA, University of Minnesota, MN, USA, University of Newcastle, New South Wales, Australia, and performed in accordance with NIH guidelines.

Generation of recombinant CaM. WT and mutant calmodulin proteins were prepared as described. Briefly, the recombinant CaM cDNA sub-cloned into a pET15b vector was mutated using QuikChange site directed mutagenesis. Proteins were expressed in E. coli BL21 (DE3) cells and purified by hydrophobic chromatography using a phenyl sepharose column. Purified protein was dialyzed overnight at 4°C twice in 50 mM HEPES at pH 7.4, 100 mM KCl, and 5 mM EGTA, and twice more with the same buffer except EGTA was lowered to 0.05 mM to remove Ca. The molecular mass of all proteins was confirmed using negative electrospray mass spectroscopy.

Measurement of Ca binding to CaM. Ca binding affinities for WT, N54I, and N98S CaM proteins were determined as described. Briefly, macroscopic binding constants for the pairs of Ca binding sites in CaM-N and CaM-C were measured by monitoring the intrinsic tyrosine and phenylalanine fluorescence of the protein over the course of a Ca titration. Free [Ca] was determined using the Ca-sensitive fluorescent indicator Fluo-5N (Invitrogen). The data were analyzed by plotting the normalized fluorescence signal vs free [Ca] and fitting to the model independent two site Adair function. The dissociation constants (Kd) for each domain are reported as the average value for the pair of sites by taking the square root of K2 from the Adair equation.

Ca wave experiments in mouse ventricular myocytes. Single ventricular myocytes from 12 to 16 weeks-old C57BL/6 mice were isolated by enzymatic digestion using a modified collagenase/protease method as described. Myocytes were permeabilized with saponin (40µg/mL) for 60 seconds and placed in internal solution composed (in mM) of K-aspartate 100, KCl 15, KH2PO4 5, MgCl2 0.75, Dextran (40,000) 4 %, HEPES 10, MgATP 5, Phosphocreatine di-Na 10, Creatine phosphokinase 10 U/ml, Glutathione (reduced) 10, and Fluo 4 0.025. Free [Ca] was 0.120 nM (calculated using MaxChelator) to allow spontaneous Ca waves. To allow equilibration of CaM binding to cellular targets, all Ca wave measurements were taken after 30 min incubation with either WT or
mutant CaM alone, or with a mixture of WT and mutant CaMs. Total free [CaM] was kept at a physiological concentration of 100 nM. Ca waves in myocytes were imaged with an LSM 510 Zeiss inverted microscope in line-scan mode. KN93 (1µM) was added to the internal solution in experiments testing the contribution of CaMKII activation on Ca waves. Ca wave analysis was carried out as described. Given the variability between different experimental days, the Ca wave frequency and Ca amplitudes data were normalized to the mean of CaM WT group obtained on the same day. For each CaM group, data were collected from at least three independent myocyte preparations. All the experiments in permeabilized myocytes were performed at room temperature.

**CaM-Kinase activation.** WT and mutant CaMs binding affinity for CaMKII was measured using a plasmid encoding for FRET-based Camui as previously described. Briefly, HEK 293 cells were cultured in a Dulbecco modified Eagle medium (Invitrogen) with 5% fetal bovine serum, 5% penicillin/streptomycin. Camui was incorporated into the HEK293 cells using a lipofectamine transfection reagent (Invitrogen). After transfection (24 h), Camui expressing cells were lysed by ultra-sonication in Ca²⁺-free buffer containing 50 mM Tris-HCl buffer (pH=7.5), 5 mM MgCl₂ and protease inhibitor cocktail. Camui activity was measured, at 37°C, in the cytosolic fraction of transfected cells in presence of 200 µM total [Ca] and WT or mutant CaMs (0 to 100 nM). WT CaM with 1 mM EGTA was used as a negative control. Fluorescence was measured after 5 min using a MS SpectraMax plate reader spectrophotometer (Molecular Devices) with excitation wavelength set at 440 nM and emission wavelengths of 477 nm (F_CFP) and 527 nm (F_YFP). Changes in FRET were assessed by F_YFP/F_CFP ratio compared to CaM-free solution.

**Single channel recording of RyR2 activity.** RyR2 was isolated from sheep hearts and incorporated into artificial lipid bilayers as previously described for rat RyR2. RyR2 gating was measured at -40 mV in cytoplasmic (vehicle) solutions containing 250 mM Cs (230mM CsCH₂O₃S + 20 mM CsCl), 2 mM ATP, 10 µM KN93 and Ca of either 0.1 or 1 µM (CaCl₂ + 5 mM BAPTA). Luminal solutions contained 250 mM Cs, 0.1 mM Ca. pH of bath solutions was buffered to pH 7.4 using 10 mM TES. A local perfusion technique that allows rapid fluid exchange
was used to apply vehicle solutions to RyR2 or vehicle plus 100 nM wt-CaM or CaM mutants. Single channel recordings were analyzed for open probability using a 50% threshold method.

**Ca spark and SR Ca content measurements.** Ca sparks were recorded in permeabilized rat ventricular cardiomyocytes (50 µg/ml saponin for 3 min). Cardiomyocytes were perfused for 30 min with internal solution containing 10 nM free [Ca] to wash-off endogenous RyR2-prebound CaM, and then free [Ca] was increased to 50 nM for 5 min prior to Ca spark recording. Ca sparks were measured using Fluo-4 (25 µM) with WT or mutant CaM (100 nM) after 30 min exposure to reach steady state. SR Ca load was assessed as the Ca transient amplitude induced by rapid application of 10 mM caffeine. The intracellular solutions contained (in mM): EGTA 0.5, HEPES 10, K-aspartate 120, free MgCl2 1, ATP 5, reduced glutathione 10, phosphocreatine di-Tris 10, creatine phosphokinase 5 U/ml, dextran (Mr: 40,000) 4%, at pH 7.2 with total [Ca] required to obtain the desired free [Ca] (using MaxChelator). Ca sparks were recorded on a confocal microscope (BioRad, Radiance 2100, 40× objective) using line scan mode with argon laser (excitation at 488 nm, emission >505 nm). Image analysis was done with ImageJ and Ca spark master.10

**Competitive inhibition of CaM binding to RyR2.** To resolve CaM binding to RyR2, we used a FRET-based assay that detects acceptor-labeled CaM (A-CaM) binding in the proximity of donor-labeled FKBP (D-FKBP) pre-targeted to RyR2.11 A-CaM denotes a single-Cys mutant of mammalian CaM (T35C) that was labeled with the thiol-reactive Alexa Fluor 568 C5 maleimide while D-FKBP denotes a single-cysteine mutant of human FKBP12.6 (T15C/C23A/C77I) that was labeled with the thiol-reactive Alexa Fluor 488 C5 maleimide, as described.12 SR vesicles from porcine ventricular myocardium (0.4 mg/ml) were incubated with D-FKBP (50 nM, 90 min, 37°C). Unbound D-FKBP was removed by centrifugation at 100,000 × g. The SR pellet containing RyR2 decorated with D-FKBP was resuspended to 10 mg/mL, and used immediately to prepare the FRET samples consisting of 3mg/ml SR, WT or arrhythmia CaM mutants (as indicated in Figure 3 of the main manuscript), 100 nM A-CaM, 20 mM K-PIPES (pH 7.0), 150 mM KCl, 5 mM GSH, 1 mM EGTA, 0.1 mg/ml BSA, 1 µg/ml Aprotinin/Leupeptin, and sufficient CaCl2 to yield the indicated free [Ca2+] (as calculated using MaxChelator, http://maxchelator.stanford.edu). After a 2.5 hr incubation at 25°C, the samples were transferred to a 384-well plate (black-wall/clear bottom), and
fluorescence spectra were read in a Gemini EM fluorescence plate reader (Molecular Devices (Sunnyvale, CA)).

FRET was calculated based on the fractional decrease of donor fluorescence ($F_D$) in the presence of acceptor ($F_{DA}$), according to $\text{FRET} = 1 - \frac{F_{DA}}{F_D}$.

**Statistics.** Data were tested for normal distribution by a Shapiro-Wilke normality test. Normally-distributed data (e.g., Ca wave frequency) were assessed using ANOVA followed by $t$-test if significant differences were found by ANOVA. Non-normally distributed data (i.e., RyR2 open probability) were analyzed using a Mann-Whitney U test for unpaired comparisons or Wilcoxon signed rank test for paired comparisons. Group differences were considered statistically significant for $P<0.05$.

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