$Ca_V1.2$ β-subunit coordinates CaMKII-triggered cardiomyocyte death and afterdepolarizations

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Excessive activation of calmodulin kinase II (CaMKII) causes arrhythmias and heart failure, but the cellular mechanisms for CaMKII-targeted proteins causing disordered cell membrane excitability and myocardial dysfunction remain uncertain. Failing human cardiomyocytes exhibit increased CaMKII and voltage-gated Ca^{2+} channel ($Ca_V1.2$) activity, and enhanced expression of a specific $Ca_V1.2$ β-subunit protein isoform (β_{2a}). We recently identified $Ca_V1.2$ β_{2a} residues critical for CaMKII phosphorylation (Thr 498) and binding (Leu 493), suggesting the hypothesis that these amino acids are crucial for cardiomyopathic consequences of CaMKII signaling. Here we show WT β_{2a} expression causes cellular Ca^{2+} overload, arrhythmia-triggering cell membrane potential oscillations called early afterdepolarizations (EADs), and premature death in paced adult rabbit ventricular myocytes. Prevention of intracellular Ca^{2+} release by ryanodine or global cellular CaMKII inhibition reduced EADs and improved cell survival to control levels in WT β_{2a}-expressing ventricular myocytes. In contrast, expression of β_{2a} T498A or L493A mutants mimicked the protective effects of ryanodine or global cellular CaMKII inhibition by reducing Ca^{2+} entry through $Ca_V1.2$ and inhibiting EADs. Furthermore, $Ca_V1.2$ currents recorded from cells overexpressing CaMKII phosphorylation- or binding-incompetent β_{2a} subunits were incapable of entering a CaMKII-dependent high-activity gating mode (mode 2), indicating that β_{2a} Thr 498 and Leu 493 are required for $Ca_V1.2$ activation by CaMKII in native cells. These data show that CaMKII binding and phosphorylation sites on β_{2a} are concise but pivotal components of a molecular and biophysical and mechanism for EADs and impaired survival in adult cardiomyocytes.

arrhythmias | calcium | calcium channel | calmodulin kinase | cardiac myocytes

The multifunctional Ca^{2+} and calmodulin-dependent protein kinase II (CaMKII) is a proarrhythmic (1) and proapoptotic (2) signaling molecule activated in failing human myocardium and in animal models of heart failure (3). The Ca^{2+} homeostatic proteins involved in excitation-contraction coupling (ECC) are CaMKII targets (4), and excessive CaMKII-mediated phosphorylation of ECC proteins has recently emerged as a critical transition event leading to myocardial dysfunction and arrhythmias (5). The L-type Ca^{2+} channel (LTCC) protein complex is the predominant entry point for Ca^{2+} that supplies intracellular sarcoplasmic reticulum (SR) Ca^{2+} stores and is an important source of inward current (I_{Ca}) for prolonging the action potential duration (APD) (6). CaMKII drives LTCCs into an active gating mode (mode 2) with frequent, prolonged openings, and mode 2 gating occurs together with an I_{Ca} property called facilitation (7). Mode 2 gating and I_{Ca} facilitation are part of a hypothesized mechanism favoring SR Ca^{2+} overload and early afterdepolarizations (EADs), arrhythmia-initiating oscillations in cell membrane potential (8). LTCCs contain a pore forming α-subunit ($Ca_V1.2$) and an accessory β-subunit protein (9), allowing use of an overexpression approach to reconstitute LTCCs with predominantly exogenous β-subunits in native cells (10, 11).

All LTCC β-subunits increase $Ca_V1.2$ opening probability (P_o), but β_{2a} is more effective than other isoforms for increasing LTCC P_o (12). The β_{2a} expression is increased in failing human hearts (12), a condition marked by increased LTCC P_o (13), APD prolongation, loss of intracellular Ca^{2+} homeostasis (14), EADs (15), and excessive cardiomyocyte death (16). Furthermore, overexpression of WT β_{2a} in adult cardiomyocytes increases I_{Ca}, induces SR Ca^{2+} overload, and stimulates apoptosis by a pathway that involves CaMKII (10), supporting the hypothesis that CaMKII phosphorylation of β_{2a} is a molecular mechanism for pathological membrane excitability and cardiomyocyte death. We recently identified CaMKII phosphorylation (Thr 498) and binding (Leu 493) sites on β_{2a}. Using this information, we tested the concept that β_{2a} Thr 498 and Leu 493 are proarrhythmic and cardiomyopathic targets for CaMKII in native adult cardiomyocytes. Here we show that β_{2a} CaMKII binding and phosphorylation sites are required for the biophysical actions of CaMKII on LTCCs and for EAD induction, whereas loss of Thr 498 or Leu 493 protects against cell death during overexpression of β_{2a}. Our findings illustrate how CaMKII phosphorylation and binding to β_{2a} can activate mode 2 gating and initiate a coherent series of integrated, but pathological cellular responses culminating in EADs and death.

Results

CaMKII Binding and Phosphorylation Sites on β_{2a} Are Critical for I_{Ca} Facilitation. Facilitation is a CaMKII-dependent, dynamic pattern of increasing peak I_{Ca} and concomitant slowing of the fast component of I_{Ca} inactivation (Fig. 1*A–F*) (17–19). We measured facilitation as the integral of inward I_{Ca} (Fig. 1*E* and *F*) elicited by a train of 15 voltage clamp steps (−80 to 0 mV, 0.5 Hz). Expression of β_{2a} WT significantly increased I_{Ca} facilitation compared to mock-infected control cells or cells expressing β_{2a} T498A or β_{2a} L493A (Fig. 1*E* and *F*). The β_{2a} L493A infected cells showed an intermediate I_{Ca} facilitation phenotype that was significantly less than in β_{2a} WT, but more than β_{2a} T498A expressing cells. SR Ca^{2+} content was significantly increased in β_{2a} WT compared

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Data deposition: The sequence reported in this paper has been deposited in the National Center for Biotechnology Information database (β_{2a} accession no. NM_053851.1; CaMKII accession no. NM_001025438.1).

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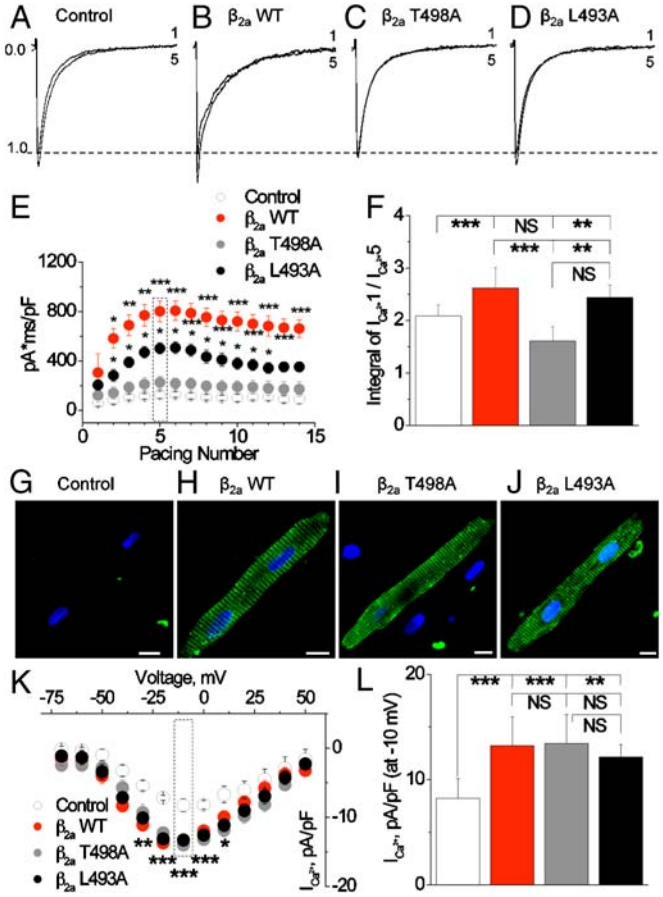

Fig. 1. CaMKII targeting to β_{2a} is required for I_{Ca} facilitation in adult cardiomyocytes. (*A–D*) Examples of the first and fifth I_{Ca} recorded in response to a train of depolarizing command steps to 0 mV from cultured adult ventricular myocytes. (*E*) Summary data for integrated I_{Ca} in response to a train of 15 voltage command pulses. (*F*) Integrated I_{Ca} from the fifth command pulse normalized to I_{Ca} from the first command pulse. These data are from *E*. Ten through 12 cells were studied for each data point in *E* and *F*. $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$ versus empty vector controls. Confocal micrographs showing cultured rabbit ventricular myocytes after infection with an empty vector (*G*) WT β_{2a}; (*H*) CaMKII-phosphorylation-resistant CaMKII β_{2a} (T498A); (*I*) CaMKII-binding-resistant β_{2a} (L493A); (*J*) β_{2a}-encoding cDNA. The green color represents immunofluorescent detection of the FLAG-tagged exogenously expressed β_{2a} subunits. Nuclei are stained blue with topro-3. (Scale bar: 10 μm.) (*K*) Current–voltage relationship showing peak L-type Ca^{2+} current (I_{Ca}) recorded from cells treated as in *G–J*. $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$ versus empty vector controls. (*L*) Peak I_{Ca} density in response to a voltage command pulse to 0 mV (data are from *K*). The vertical ticks mark groups for statistical comparison.

to β_{2a} mutant infected cells (Fig. S1). These findings suggest that CaMKII binding and phosphorylation sites are critical for β_{2a}-mediated increases in I_{Ca} facilitation and Ca^{2+} entry that contribute to SR Ca^{2+} loading in adult ventricular myocytes.

WT and CaMKII Resistant β_{2a} Mutants Show Normal Membrane Targeting. Confocal immunofluorescent detection of exogenous β_{2a} showed that β_{2a} WT, β_{2a} T498A, and β_{2a} L493A had a repetitively spaced pattern of expression (Fig. 1*G–J*), consistent with the known enrichment of native $Ca_V1.2$ β-subunits in adult ventricular myocyte T-tubular membranes (20). The ratio of peak I_{Ca} to peak SR Ca^{2+} release during cardiac ECC requires precise localization of sarcolemmal $Ca_V1.2$ and SR ryanodine receptor Ca^{2+} release channels. In order to test if the exogenous β_{2a} subunits are positioned to support ECC, we measured the ratio of peak intracellular Ca^{2+} release triggered by I_{Ca} in voltage-clamped cells, under conditions that controlled peak I_{Ca} (by post hoc selection) and matched SR Ca^{2+} content using a genetically individualized conditioning protocol (*SI Materials and Methods*). Exogenous β_{2a} subunits resulted in a lower ECC gain compared to mock-infected control cells (Fig. S2), potentially suggesting that β_{2a} overexpression alters the relationship between $Ca_V1.2$ and ryanodine receptors in adult cardiomyocytes. However, there were no differences in ECC gain at any voltage command step between β_{2a} WT, β_{2a} T498A, or β_{2a} L493A infected cells. Thus, the selective loss of I_{Ca} facilitation in T498A and L493A β_{2a} infected cells indicates that concise but fundamental biophysical defects occur in LTCC channels assembled with a CaMKII binding- or phosphorylation-disabled β_{2a} subunit.

β_{2a} Leu 493 and Thr 498 Are Required for CaMKII Activation of Mode 2 Gating. CaMKII increases mode 2 gating in LTCCs (7), but the molecular targets are unknown. In order to assess the effects of T498A and L493A mutants on LTCCs, we measured single $Ca_V1.2$ channel currents from intact infected myocytes. We identified two populations of $Ca_V1.2$ opening times (Fig. 2*A–F*). The duration of the short (Fig. 2*E*) and long (Fig. 2*F*) $Ca_V1.2$ opening times were not different between the experimental groups. However, β_{2a} T498A mutants exhibited a significantly reduced relative frequency of longer opening times and reduced mean P_o (Fig. 2*H*) compared to cells infected with β_{2a} WT, whereas

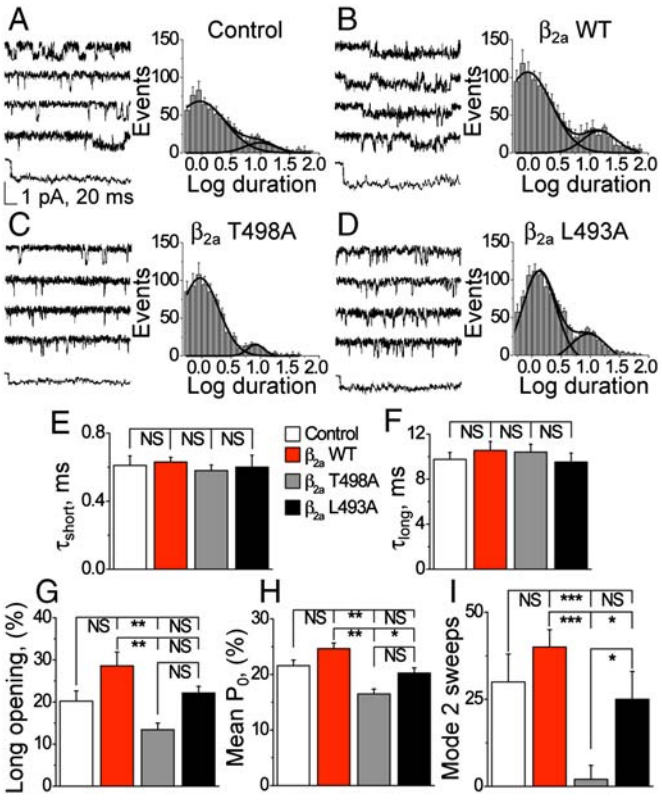

Fig. 2. Reduced opening probability (P_o) and mode 2 gating events in LTCC from β_{2a} WT compared to T498A or L493A expressing ventricular myocytes. Single LTCC current tracings and modal gating analysis from (*A*) mock-infected control cells, (*B*) β_{2a} WT infected cells, (*C*) β_{2a} T498A infected cells, and (*D*) β_{2a} L493A infected cells. Each panel (*A–D*) shows representative single LTCC current recordings and an ensemble tracing averaged from 40 sweeps (*Left*). A histogram in each panel shows that LTCC openings partition into short and long opening times (*Right*). The time constants (τ) for the short (*E*) and long (*F*) opening times are not different between experimental groups. In contrast, the percent of long openings (*G*), mean Po (*H*), and sweeps with mode 2 activity (*I*) are significantly less in β_{2a} T498A compared to β_{2a} WT infected cells. Color-coded legends are as in Fig. 1 and statistical comparisons between groups are denoted as in Fig. 1*F*. Eight through 10 cells in each group gave 250–300 active sweeps and were used for each data point in *E–I*.

the frequency of long opening times (Fig. 2*G*) and mean P_o (Fig. 2*H*) were not different between β_{2a} T498A and β_{2a} L493A. We next grouped $Ca_V1.2$ openings into gating modes, as previously described (7). These analyses revealed that LTCCs expressing β_{2a} WT had significantly more mode 2 gating activity than LTCCs recorded from cells with β_{2a} T498A or β_{2a} L493A expression (Fig. 2*I*).

We compared LTCC gating responses to global cellular CaMKII inhibition, by expression of a CaMKII inhibitory peptide (CaMKIIN) or shRNA knock down of CaMKIIδ (Fig. S3), in β_{2a} WT expressing and mock-infected control cells. CaMKII inhibition significantly reduced I_{Ca} facilitation in control and WT β_{2a} overexpressing cells, and significantly reduced the frequency of long LTCC opening times and P_o compared to WT β_{2a} expression or mock-infected control cells without CaMKII inhibition (Fig. S4). Finally, CaMKII inhibition with WT β_{2a}-infected or mock-infected control cells significantly reduced the frequency of mode 2 gating to levels present in β_{2a} T498A expressing ventricular myocytes (compare Fig. S4*G* to Fig. 2*I*). These data show that LTCCs recorded under conditions of global CaMKII inhibition or lacking the β_{2a} CaMKII Thr 498 phosphorylation site, but in the presence of normal CaMKII activity, exhibit similar amounts of mode 2 gating.

To further test the concept that β_{2a} Thr 498 and Leu 493 were crucial for CaMKII-induced mode 2 gating, we designed experimental conditions where CaMKII activity was directly controlled. We measured $Ca_V1.2$ single channel currents from membrane patches excised from noninfected cultured ventricular myocytes and from ventricular myocytes infected with β_{2a} WT, β_{2a} T498 or β_{2a} L493A. We enriched the bath (cytoplasmic) solution with exogenous, constitutively active CaMKII to test the ability of native and β_{2a} WT, β_{2a} T498A, and β_{2a} L493A reconstituted channels to respond to CaMKII (Fig. S5). Mode 2 gating was observed significantly less in β_{2a} T498A ($P < 0.001$ versus mock-infected controls and β_{2a} WT) and β_{2a} L493A ($P < 0.05$ versus mock-infected controls and $P < 0.001$ versus β_{2a} WT) compared to β_{2a} WT or endogenous channels. We interpret these findings to mean that CaMKII actions on LTCCs are predominantly controlled by β_{2a} Thr 498 and Leu 493. Because Thr 498 is conserved on all major β-subunit isoforms in heart (21), it seems likely that this Thr is similarly critical for CaMKII effects on all $Ca_V1.2$ complexes.

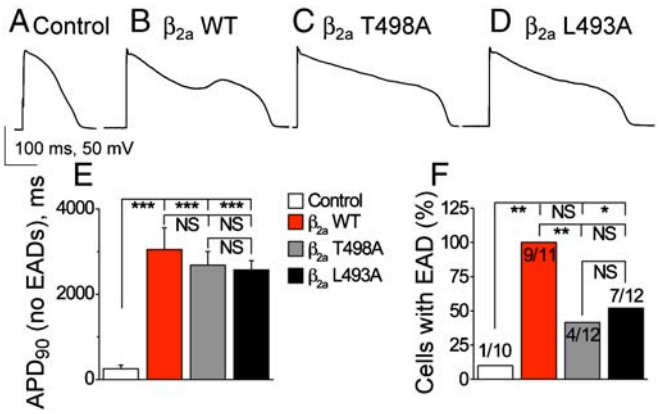

Fig. 3. CaMKII sites are required for frequent EADs but not for APD prolongation. Representative action potentials recorded from (*A*) mock-infected control cells, (*B*) β_{2a} WT infected cells, (*C*) β_{2a} T498A infected cells, and (*D*) β_{2a} L493A infected cells. (*E*) APD recorded at 90% repolarization to baseline (APD_{90}) are significantly and equivalently increased in all groups. (*F*) EAD frequency is significantly increased over mock-infected cells only in β_{2a} WT infected cells, while β_{2a} T498A and L493A infected cells are protected. Color-coded legends are as in Fig. 1 and statistical comparisons between groups are denoted as in Fig. 1*F*. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Loss of CaMKII Sites on β_{2a} Reduces EADs During APD Prolongation. Expression of β_{2a} WT, β_{2a} L493A, and β_{2a} T498A significantly and equivalently prolonged the APD compared to control vector only infected ventricular myocytes (Fig. 3*A–E*). The striking increases in APD are consistent with the known potential for I_{Ca} to slow membrane repolarization in adult ventricular myocytes (6). APD prolongation by β_{2a} WT expression favored EADs, but APD prolongation by β_{2a} T498A expression did not significantly increase EADs compared to control (Fig. 3*F*). β_{2a} L493A expressing cells showed a reduction in EAD frequency compared to β_{2a} WT expressing cells that was not significantly different than β_{2a} T498A expressing cells (Fig. 3*F*). Cells with global CaMKII inhibition and infected with β_{2a} WT were also resistant to EADs, despite significant APD prolongation (Fig. S6). These results indicate a reduced proarrhythmic potential from APD prolongation due to β_{2a} expression lacking Leu 493 or Thr 498, and suggest that CaMKII binding and phosphorylation sites on β_{2a} are required for efficient EAD induction during APD prolongation in adult ventricular myocytes.

SR Ca^{2+} Release and CaMKII Effects on β_{2a} Contribute to Cytotoxicity. Ventricular myocytes with β_{2a} WT expression die prematurely by a CaMKII-dependent process (10). We paced cultured ventricular myocytes and measured cell viability at various time points over 24 h (Fig. 4*A* and *B*) to test if CaMKII regulated cardiomyocyte survival through β_{2a} Leu 493 or Thr 498. β_{2a} WT infected cell cultures showed a significantly higher proportion of dead cells, assessed by morphological criteria (Fig. 4*B*) or Trypan blue exclusion (Fig. S7*A* and *B*), after 3 h of pacing, compared to β_{2a} T498A or vector control infected ventricular myocytes. The β_{2a} L493A

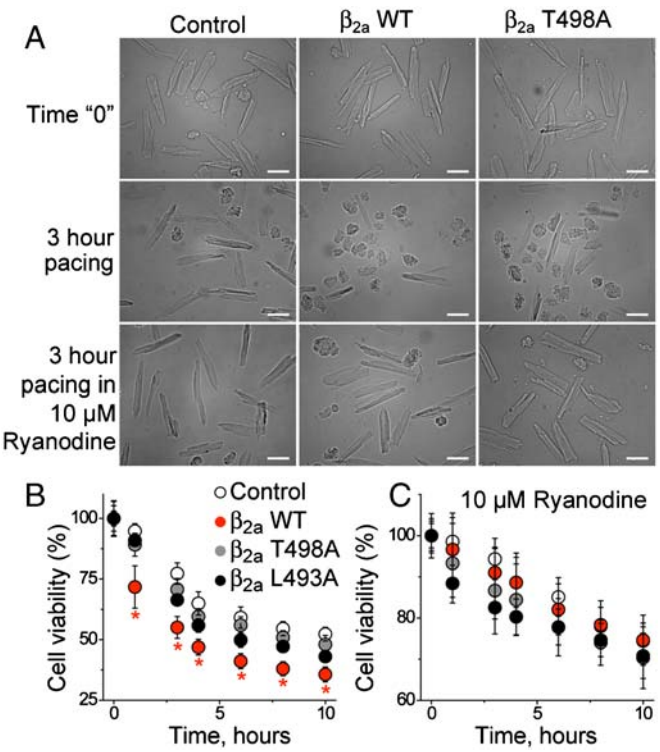

Fig. 4. Maximal survival by ryanodine treatment and improved survival with β_{2a} T498A and L493A compared to WT expressing cardiomyocytes. (*A*) Representative micrographs of mock-infected, β_{2a} WT and β_{2a} T498A infected ventricular myocytes (left to right) at baseline and after 3 h of pacing in the absence and presence of ryanodine (top to bottom). (Calibration bar: 50 μm.) Summary data for cell viability based on morphological criteria in the absence (*B*) or presence (*C*) of ryanodine (10 μM). Each data point is from an average of ≥50 cells. $^*P < 0.05$ compared to vector-infected controls. Color-coded legends in *B* and *C* are as in Fig. 1.

expressing ventricular myocytes showed an intermediate viability pattern that was not significantly different than for β_{2a} T498A infected cells (Fig. 4*B*). Loss of cell viability was similar in all groups after pacing for 24 h. CaMKII enhances SR Ca^{2+} release (5) and preventing SR Ca^{2+} release improves viability in ventricular myocytes expressing β_{2a} WT (10). In order to test if reduced survival by β_{2a} WT expression required SR Ca^{2+} release in our model, we repeated the cell viability assays in ventricular myocytes infected with each of the β_{2a} constructs in the presence of ryanodine (10 μM). Ryanodine treatment abolished the differences between genotypes and significantly increased the percentage of viable cells at each time point (Fig. 4*C*). Finally, we measured cardiomyocyte viability in response to pacing during global cellular CaMKII inhibition in the absence (Fig. S7*C*) or presence of ryanodine (Fig. S7*D*). Surprisingly, global CaMKII inhibition or ryanodine treatment equivalently improved viability to control levels for β_{2a} WT expressing cells at each of the time points. These data suggest a model where CaMKII phosphorylation of β_{2a} can initiate a pathological cascade of enhanced cellular Ca^{2+} entry causing excessive SR Ca^{2+} release that leads to reduced cardiomyocyte survival.

SR Ca^{2+} Release Enhances EADs by Activating CaMKII Targeted to β_{2a}. We repeated the AP recordings in the presence of ryanodine to test if reduced SR Ca^{2+} release affected APD or EADs. Ryanodine significantly prolonged APD but reduced EADs (Fig. S7*E* and *F*) to levels previously observed in β_{2a} T498A expressing cells (Fig. 3*F*). EAD suppression by ryanodine suggested that SR Ca^{2+} release is critical for activation of β_{2a}-targeted CaMKII. To test this concept, we infected adult cardiomyocytes with a constitutively active T286D CaMKIIδ mutant. We observed APD prolongation and spontaneous EADs in cells infected with constitutively active CaMKII (Fig. S8), but EADs in the T286D CaMKIIδ infected cells were not inhibited by ryanodine (Fig. S8*C*). The $Ca_V1.2$ antagonist nifedipine (500 nM) eliminated EADs and significantly and equivalently shortened APDs in myocytes infected with the T286D CaMKIIδ mutant (Fig. S8*D*), suggesting that LTCCs were critical for the proarrhythmic actions of CaMKII. In order to establish the role of β_{2a} L493 and T498 in transducing EADs by constitutively active CaMKII, we coinfected cardiomyocytes with T286D CaMKIIδ and each of the β_{2a} constructs. CaMKIIδ T286D caused EADs in 9/10 control cells, but was significantly less effective at inducing EADs in myocytes infected with β_{2a} L493A or T498A (Fig. S8*E*), despite similar APD prolongation in each of these groups (Fig. S8*F*). We interpret these findings to mean that SR Ca^{2+} release is necessary for activating CaMKII that ultimately triggers EADs by targeting β_{2a} L493 and T498. Constitutively active CaMKII expression circumvents the need for activating SR Ca^{2+}, but nevertheless requires β_{2a} L493 and T498 in order to effectively induce EADs.

New Mathematical Model of Increased Mode 2 Gating and EADs. Our data support a model where a single $Ca_V1.2$ phosphorylation event at β_{2a} Thr 498 can initiate a cascade of CaMKII-triggered cellular events orchestrated by ECC proteins, including increased $Ca_V1.2$ mode 2 gating, augmented I_{Ca} facilitation, and cellular Ca^{2+} entry, increased SR Ca^{2+} release and EADs. We developed a mathematical model of CaMKII regulation of ECC proteins that incorporates our experimental findings (Fig. S9). Computer simulations in the Luo-Rudy ventricular cell model show that β_{2a} overexpression results in greater CaMKII activity during the action potential plateau compared to the T498A mutant. CaMKII activation, in turn, increases the number of LTCCs exhibiting mode 2 gating, which promotes reactivation of the I_{Ca} and EADs during AP repolarization. Consistent with our experimental observations, block of SR Ca^{2+} release eliminates EADs by reducing CaMKII and mode 2 gating activity. To determine whether the protective effects of the T498A mutant required reduced SR Ca^{2+} content (Fig. S1), we computed T498A and WT action potentials under reversed steady-state SR Ca^{2+} loading conditions (relatively high SR $[Ca^{2+}]$ in T498A and low SR $[Ca^{2+}]$ in WT). These computer simulations demonstrate that WT β_{2a} expressing cells develop EADs, whereas T498A mutant expressing cells are resistant to EADs (Fig. S9), independent of SR Ca^{2+} load, suggesting that EADs were ultimately dependent on Thr 498 phosphorylation and consistent with our findings in ryanodine-treated cells expressing constitutively active CaMKII (Fig. S8).

Discussion

β_{2a}-Initiated Molecular Pathway for EADs and Cell Death. Our results indicate that expression of WT β_{2a} is highly effective at leveraging the ECC protein machinery in adult ventricular myocytes by connecting cellular Ca^{2+} entry with excessive SR Ca^{2+}, EADs, and impaired cell survival. Arrhythmias and heart failure occur together, in part, because they are both consequences of hyperphosphorylated ECC proteins (22). CaMKII catalyzes phosphorylation of each of the major Ca^{2+} homeostatic proteins, which regulate cellular Ca^{2+} entry and SR Ca^{2+} uptake and release. CaMKII catalyzes the phosphorylation of β Thr 498 (11), additional sites on the $Ca_V1.2$ α-subunit (23, 24), phospholamban (25), and ryanodine receptors (26). $Ca_V1.2$ is a critical control point for cellular Ca^{2+} entry in ventricular myocardium and this Ca^{2+} entry due to I_{Ca} ultimately sustains the SR Ca^{2+} content necessary for SR Ca^{2+} release and myofilament contraction. Thus, the hyperactive $Ca_V1.2$ channels that occur in failing human ventricular myocytes (13) are well positioned to provoke EADs and intracellular Ca^{2+} overload. Expression of β_{2a} increases in heart failure, relative to other β-subunit isoforms (12), while expression of ryanodine receptors and the phospholamban-regulated SR Ca^{2+} ATPase (SERCa2a) are generally reduced or unchanged in failing myocardium (27). Studies in a rabbit model of heart failure show increased CaMKII activation, afterdepolarizations, arrhythmias, and sudden death, but without an increase in peak I_{Ca} (28). Thus, CaMKII signaling to the ryanodine receptor (28) and other ion channels is also important for arrhythmias in structural heart disease. Our findings are consistent with a concept that CaMKII actions at β_{2a} can initiate and orchestrate CaMKII-dependent actions on membrane excitability and cell survival that are important for heart failure progression, because loss of CaMKII binding or phosphorylation sites completely disrupts the connection between β_{2a} WT expression and EADs and partially protects against premature cell death.

β-Subunit is Critical for CaMKII Signaling to $Ca_V1.2$. Our data show that CaMKII targeting β_{2a} is preeminent over other CaMKII actions at $Ca_V1.2$ for modulating LTCC mode 2 gating, I_{Ca} facilitation, and EADs in native adult cardiomyocytes. Although our findings do not rule out a role for CaMKII phosphorylation of other LTCC sites, including amino acids identified on $Ca_V1.2$ (23, 24), they strongly suggest that Thr 498 and Leu 493 on β_{2a} are dominant over other CaMKII regulatory LTCC sites in native adult heart cells. Our model uses overexpression to probe the role of CaMKII at β_{2a} for regulating membrane excitability and cell survival, and it is possible that β_{2a} overexpression could bias our findings toward LTCC and away from alternative CaMKII ECC protein targets. However, in our model, expression of all β_{2a} backgrounds and peak I_{Ca} were closely matched, allowing us to assign clear roles to Thr 498 and Leu 493 for $Ca_V1.2$ gating and facilitation.

What is the Relationship Between APD and Arrhythmias? Our experimental and computational data add to a growing body of evidence suggesting that CaMKII activation is a critical step linking increased APD with EADs and arrhythmias. Rabbits with prolongation of the electrocardiographic QT interval showed significantly reduced proarrhythmia in the presence of systemic

calmodulin inhibition (29), and Langendorff-perfused rabbit hearts with APD prolongation had reduced EAD induction during infusion of calmodulin or CaMKII inhibitory drugs (30). In these studies, calmodulin and CaMKII inhibition suppressed arrhythmias and EADs without significantly affecting QT interval or APD, suggesting that APD prolongation, *per se*, was insufficient for proarrhythmia in vivo. Our present studies provide fresh molecular details to the concept that CaMKII enables APD prolongation-dependent proarrhythmia by showing that the APD prolongation due to increased I_{Ca} requires SR Ca^{2+} to activate CaMKII and that activated CaMKII binds to and phosphorylates β_{2a} to trigger EADs.

Materials and Methods

Ventricular Myocyte Isolation and Viral Infection. The cardiomyocyte isolation from adult male New Zealand white rabbits and viral infections were performed as described with minor modifications (31). Virus was added to the cells at a multiplicity of infection of 1–3, and cell cultures were maintained for 24–36 h. See *SI Materials and Methods* for full details.

β-subunits, CaMKII Protein and CaMKII Inhibition. Expression of β-subunits (11, 21), CaMKII protein, and CaMKII inhibition were performed according to our published methods. See *SI Materials and Methods* for full details (7).

Confocal Microscopy and Immunofluorescence. Confocal Ca^{2+} measurement and immunofluorescent studies were performed according to established methods (11), with minor modifications. See *SI Materials and Methods* for full details.

Electrophysiology. Whole cell mode current and voltage clamp studies, and single channel LTCC recordings, were performed using our previously published methods, with minor modifications. We selected β_{2a} infected cells with peak I_{Ca} 13–16 pA/pF and mock-infected cells with peak I_{Ca} from 6–8 pA/pF for analysis in current clamp and whole cell mode voltage clamp studies to avoid potential confounding effects of variable $Ca_V1.2$ expression amongst the β_{2a}-infected cells. See *SI Materials and Methods* for full details.

Cell Pacing and Viability Measurements. Dissociated ventricular myocytes were cultured on a 12-mm-diameter (glass #1) cover glasses placed into four-well Nunclon Delta Treated dishes (10 coverslips in each well). We used morphology and Trypan blue staining to estimate cell viability (10). See *SI Materials and Methods* for full details.

Modeling. The Luo-Rudy model of the mammalian ventricular action potential including a Markov representation of I_{Ca} is used as the basis for all computer simulations. See *SI Materials and Methods* for full details.

Statistical Analysis. Data are presented as mean ± SEM. *P* values were assessed with a paired Student's *t* test or ANOVA, as appropriate, for continuous data. The Bonferroni test was used for post hoc testing. The Fisher exact test was used to analyze EAD induction frequency. The null hypothesis was rejected for $P \leq 0.05$.

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