Protection from Cardiac Arrhythmia Through Ryanodine Receptor-Stabilizing Protein Calstabin2

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thus envision the part of the pore that traverses the plasma membrane as a barrel formed by several copies of the transmembrane segment of Syx, arranged in parallel to form a complete circle (Fig. 4E). It is also possible that other molecules, either protein or lipid, intercalate between the Syx segments to complete the pore structure. Other molecules could provide a connection to the extracellular fluid if the C terminus of Syx remains buried in the membrane (*27*) even after the fusion pore opens. Although the present results provide no direct evidence regarding the participation of other proteins, the observation that the synaptic SNARE proteins, Syx, SNAP-25, and synaptobrevin/vesicle-associated membrane protein (VAMP) constitute a minimal machine for liposome fusion (*23*) suggests that Syx may be the only molecular component of the fusion pore in the plasma membrane. Extrapolating the present results to the vesicle membrane suggests that the transmembrane segment of synaptobrevin/VAMP forms the complementary fusion-pore structure through the vesicle membrane, and that the SNARE complex holds these two parts together. The SNARE complex would then have to form at some point before the fusion pore opens and neurotransmitters are released (*28*, *29*).

The cooperative participation of SNARE complexes in fusion (*30*, *31*) supports the idea that fusion pores are formed from multiple copies of Syx. With a barrel of Syx transmembrane segments as the minimal structure, we constructed a simple model of the plasma membrane side of the fusion pore (Fig. 4E). This model yielded copy numbers ranging from 5 to 8 depending on whether we used the contact points (Fig. 4E, left) or innermost points (Fig. 4E, right) of the transmembrane segments, respectively (*32*). The inclusion of other molecules in the pore structure could reduce the number of participating Syx molecules. Variations in fusion-pore size (*6*, *7*, *11*) could reflect different molecular partners or different numbers of Syx transmembrane segments.

Because fusion-pore opening and dilation constitute critical steps in exocytosis, hypotheses for regulation by Ca^{2+} require that a Ca^{2+} sensor targets the proteins that form the fusion pore. The modulation of these steps by synaptotagmin, the putative Ca^{2+} sensor of exocytosis, therefore requires interactions with fusion-pore proteins. This indicates that the regulation of fusion-pore stability by synaptotagmin (*10*, *11*) is likely to be mediated by Ca^{2+}-promoted binding to SNAREs or SNARE complexes (*12*, *13*).

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25. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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32. We used a simple formula for channel conductance: $\gamma = A/\rho l$ (*33*). γ was taken as 100 pS from our measurement for wild-type Syx (Fig. 4D). ρ is the resistivity of the pore lumen (100 ohm·cm), A is the area, and l is the length. $l \sim 10$ nm because it spans two lipid bilayers. Solving for A gave 1 nm^2. This gives a circle radius of 0.56 nm. If each transmembrane segment is an α helix with a radius of 0.35 nm, we can arrange 5 to 6 segments in a circle for which the points of contact form a circle with a 0.56-nm radius (Fig. 4E, left), or 8 segments for which the innermost points form a circle with a 0.56-nm radius (Fig. 4E, right).
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Protection from Cardiac Arrhythmia Through Ryanodine Receptor–Stabilizing Protein Calstabin2

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Ventricular arrhythmias can cause sudden cardiac death (SCD) in patients with normal hearts and in those with underlying disease such as heart failure. In animals with heart failure and in patients with inherited forms of exercise-induced SCD, depletion of the channel-stabilizing protein calstabin2 (FKBP12.6) from the ryanodine receptor–calcium release channel (RyR2) complex causes an intracellular Ca^{2+} leak that can trigger fatal cardiac arrhythmias. A derivative of 1,4-benzothiazepine (JTV519) increased the affinity of calstabin2 for RyR2, which stabilized the closed state of RyR2 and prevented the Ca^{2+} leak that triggers arrhythmias. Thus, enhancing the binding of calstabin2 to RyR2 may be a therapeutic strategy for common ventricular arrhythmias.

Ventricular tachyarrhythmias that cause SCD are often associated with common heart diseases, such as heart failure, but may also occur in individuals without structural heart disease (*1*). Treatment remains largely empirical, in part because of an incomplete understanding of the underlying cellular mechanisms that trigger the arrhythmias (*2*). RyR2 is required for Ca^{2+} release from the sarcoplasmic reticulum (SR) during systole, when activation of heart muscle contraction occurs. During the resting phase of the cardiac cycle (diastole), binding of calstabin2 (also known as the FK506-binding protein, FKBP12.6) to RyR2 helps maintain the channel in a closed state to prevent leakage of SR Ca^{2+} into the cytoplasm (*3*, *4*). In heart failure (*5*) and catecholaminergic polymorphic ventricular tachycardia (an inherited form of exercise-

induced ventricular arrhythmias) (*6*), depletion of calstabin2 from the RyR2 macromolecular complex results in "leaky" RyR2 channels that contribute to both diseases. Aberrant Ca^{2+} release from the SR during diastole through leaky cardiac RyR2 can result in diastolic changes in membrane potential (e.g., delayed afterdepolarizations, DADs) and is a molecular "trigger" for ventricular arrhythmias (*6*). Calstabin2-deficient mice (*calstabin2* $^{-/-}$) consistently exhibit exercise-induced ventricular arrhythmias (*6*). Accordingly, restoring binding of calstabin2 to the RyR2 complex to prevent aberrant Ca^{2+} leakage from the SR is a potential molecular approach to preventing arrhythmias (*7*, *8*). The experimental drug JTV519, a 1,4-benzothiazepine derivative, has recently been shown to reduce diastolic SR Ca^{2+} leak in an animal model of heart failure (*9*, *10*). In the present study, we examined its efficacy and mechanism of action in a cardiac arrhythmia model. Initial attempts to prepare gram quantities of JTV519 {4-[3-(4-benzylpiperidin-1-yl) propionyl]-7-methoxy-2,3,4,5-tetrahydro-1,4-benzothiazepine monohydrochloride} through the 1,4-benzothiazepine intermediate 7-methoxy-2,3,4,5-tetrahydro-1,4-benzothiazepine (compound **6** in fig. S1) were unsuccessful (*11*), so we developed an alternative synthesis (*12*).

Calstabin2$^{-/-}$ mice exhibit ventricular arrhythmias and SCD after strenuous exercise if injected with a low dose of epinephrine (*6*). The hearts of *calstabin2*$^{+/-}$ or *calstabin2*$^{-/-}$ mice were structurally normal as assessed by echocardiography and histology (*6*, *13*). Untreated conscious *calstabin2*$^{+/-}$ mice, as well as JTV519-treated (7-day continuous infusion 0.5 mg/kg body weight per hour) *calstabin2*$^{+/-}$ and *calstabin2*$^{-/-}$ mice, revealed no significant differences in electrocardiographic (ECG) parameters, namely, heart rate (RR), conduction intervals (PR, QRS), or repolarization intervals (rate-corrected QT interval; QTc) (Fig. 1A and table S1).

To test for cardiac arrhythmias, *calstabin2*$^{+/-}$ and *calstabin2*$^{-/-}$ mice were exercised according to a stress-inducing protocol (*6*, *14*). Whereas 100% of *calstabin2*$^{+/-}$ mice displayed ventricular tachyarrhythmias (VT) or syncopal events during the time they were subjected to the protocol, none of the *calstabin2*$^{+/-}$ mice pretreated with JTV519 developed arrhythmias or syncopal events (Fig. 1, B and C). Moreover, 89% of *calstabin2*$^{+/-}$ mice died during or after exercise, whereas none of the JTV519-treated *calstabin2*$^{+/-}$ mice died. In contrast to treated *calstabin2*$^{+/-}$ mice, 100% of the *calstabin2*$^{-/-}$ mice treated with JTV519 developed VT during the period of the protocol and died despite the JTV519 treatment (Fig. 1C). Taken together, these data suggest that calstabin2 may be required for the antiarrhythmic protection by JTV519.

To further characterize the antiarrhythmic properties of JTV519, we subjected wild-type, *calstabin2*$^{+/-}$, and *calstabin2*$^{-/-}$ mice to programmed electrical stimulation protocols. After injection with isoproterenol (0.5 mg/kg), VTs were induced by rapid overdrive pacing in 71% of *calstabin2*$^{+/-}$ mice but not in wild-type mice ($P < 0.05$) (Fig. 2B). *Calstabin2*$^{+/-}$ mice pretreated with JTV519 (0.5 mg/kg per hour) were significantly less susceptible than untreated calstabin2$^{+/-}$ mice to overdrive pacing–induced VTs ($P < 0.05$). In contrast, 67% of *calstabin2*$^{-/-}$ mice treated with JTV519 developed VTs during overdrive pacing. VTs could be induced with a single premature beat in 71% of *calstabin2*$^{+/-}$ mice (Fig. 2, A and B). No VTs were observed in *calstabin2*$^{+/-}$ mice treated with JTV519 when we used a single–premature beat protocol (Fig. 2A). Using a more aggressive pacing protocol, involving two consecutive off-cycle beats (S1-S2-S3), we induced VTs in 100% of untreated *calstabin2*$^{+/-}$ mice (Fig. 2B). Treatment with JTV519 completely eliminated inducible VTs in *calstabin2*$^{+/-}$ mice. Although JTV519 has been reported to inhibit voltage-gated Ca^{2+} and K^+ channels at the concentrations used in our experiments (*15*), JTV519 treatment did not prevent VTs in *calstabin2*$^{-/-}$ mice; this finding indicates that the ability of

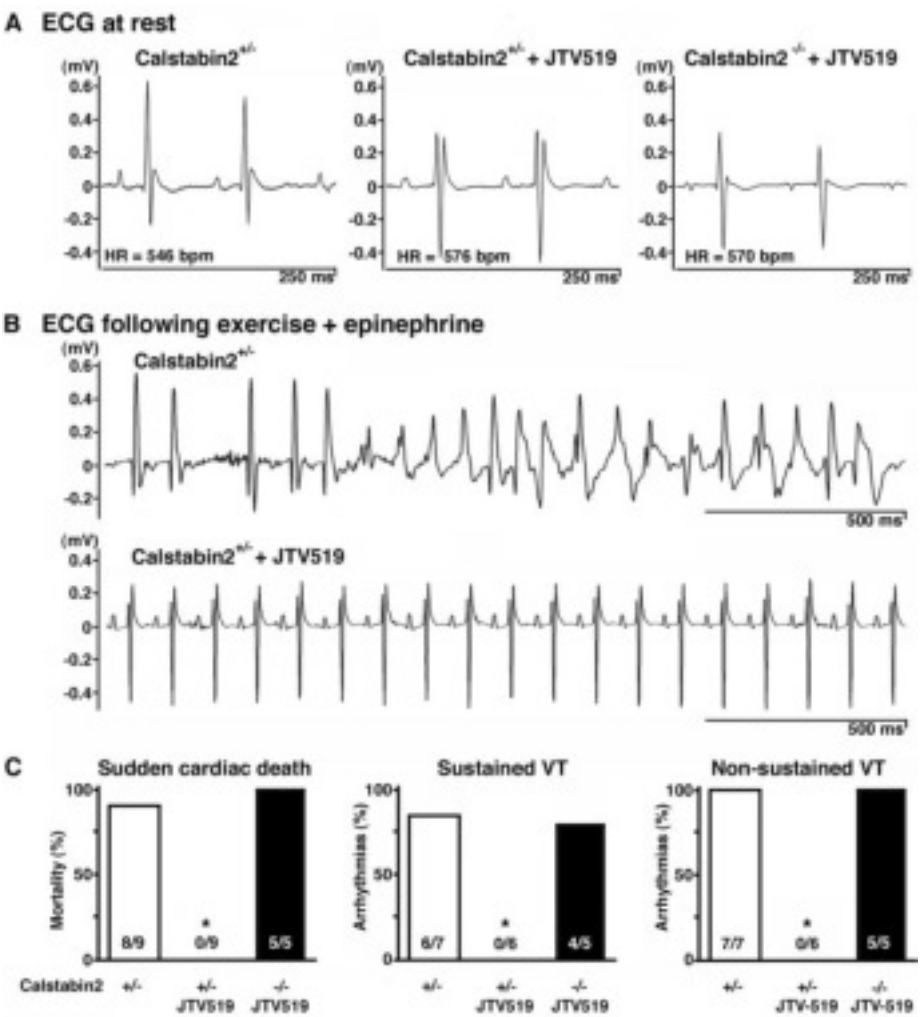

Fig. 1. Prevention of exercise-induced ventricular arrhythmias by JTV519 in *calstabin2* $^{+/-}$ mice. (**A**) Representative ECGs of an untreated *calstabin2*$^{+/-}$ mouse and JTV519-treated *calstabin2*$^{+/-}$ and *calstabin2*$^{-/-}$ mice. Mice were treated with 0.5 mg JTV519/per kilogram of body weight per hour for 7 days with an implanted osmotic mini-pump. JTV519 had no effect on resting heart rate or other ECG parameters. HR, heart rate. (**B**) Sustained polymorphic ventricular tachycardia recorded by telemetry in an untreated *calstabin2*$^{+/-}$ mouse (upper tracing) subjected to exercise testing, immediately followed by injection with 0.5 mg epinephrine per kilogram of body weight. Representative telemetry ECG recording of a JTV519-treated *calstabin2*$^{+/-}$ mouse following the same protocol (bottom tracing). (**C**) Numbers of mice with cardiac death (left), sustained VTs (>10 beats, middle), and nonsustained VTs (3 to 10 arrhythmogenic beats, right) in experimental groups of mice subjected to exercise testing and injection with 0.5 mg/kg epinephrine.

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JTV519 to induce rebinding of calstabin2 to RyR2 underlies its antiarrhythmic actions.

Ryanodine receptor–calcium release channels are tetramers comprising four RyR2 monomers, each of which binds a single calstabin2 molecule. Calstabin2 stabilizes the RyR2 channel in the closed state (*6*, *16*). Haploinsufficiency of calstabin2 resulted in a reduction (~40%) in the amount of calstabin2 protein in cardiac lysates compared with that in lysates from *calstabin2*$^{+/+}$ hearts (fig. S2A). The amount of calstabin2 protein in the RyR2 channel complex of *calstabin2*$^{+/-}$ mice was ~65% of that in *calstabin2*$^{+/+}$ mice (fig. S2B).

Phosphorylation by cyclic adenosine 5′-monophosphate–dependent protein kinase A (PKA) of RyR2 at Ser^{2809} causes dissociation of calstabin2 from the channel (*5*). Pretreatment of animals with JTV519 (0.5 mg/kg per hour) did not affect phosphorylation of Ser^{2809} in *calstabin2*$^{+/-}$ or *calstabin2*$^{-/-}$ mice (Fig. 3, A and B). Compared with channel complexes from *calstabin2*$^{+/+}$ mice, RyR2 complexes from *calstabin2*$^{+/-}$ mice were significantly more depleted of calstabin2 after exercise (Fig. 3, A and B). Treatment with JTV519, however, prevented the loss of calstabin2 from the RyR2 macromolecular complex in *calstabin2*$^{+/-}$ mice during exercise (Fig. 3, A and B).

The probabilities that channels will be open (P_o) for RyR2 channels from *calstabin2*$^{+/-}$ mice subjected to exercise were significantly increased compared with those of channels from exercised *calstabin2*$^{+/+}$ mice. Treatment of exercised *calstabin2*$^{+/-}$ mice with JTV519 (0.5 mg/kg per hour) significantly reduced the channel P_o compared with that of channels from exercised mice that were not treated (Fig. 3, C and D), consistent with increased amounts of calstabin2 in the RyR2 complex (Fig. 3, A and B). In contrast, JTV519 treatment of exercised *calstabin2*$^{-/-}$ mice did not result in channels with a low P_o (Fig. 3, C and D). We examined RyR2 single channels in the presence of low cytosolic [Ca^{2+}] (150 nM), using Ca^{2+} as the charge carrier. These conditions mimic those of the heart during diastole, when the RyR2 channels should have a low P_o to prevent diastolic Ca^{2+} leak from the SR, which can trigger cardiac arrhythmias. The significant reduction in the RyR2 P_o observed in JTV519-treated, exercised *calstabin2* $^{+/-}$ mice suggests that these channels will not become leaky during diastole, consistent with the finding that no arrhythmias were observed.

Exercise is associated with increased phosphorylation of RyR2 by PKA, which leads to loss of calstabin2 from the channel macromolecular complex (*6*). To further examine the mechanism by which JTV519 prevents VTs, we simulated the exercise conditions using PKA phosphorylation of membrane preparations of HEK 293 cells expressing wild-type RyR2 (RyR2-WT) channels. PKA-phosphorylated RyR2 channels were incubated with calstabin2 (250 nM) in the presence of various concentrations of JTV519. Incubation of PKA-phosphorylated RyR2-WT channels and calstabin2 with 100 nM or 1.0 μM

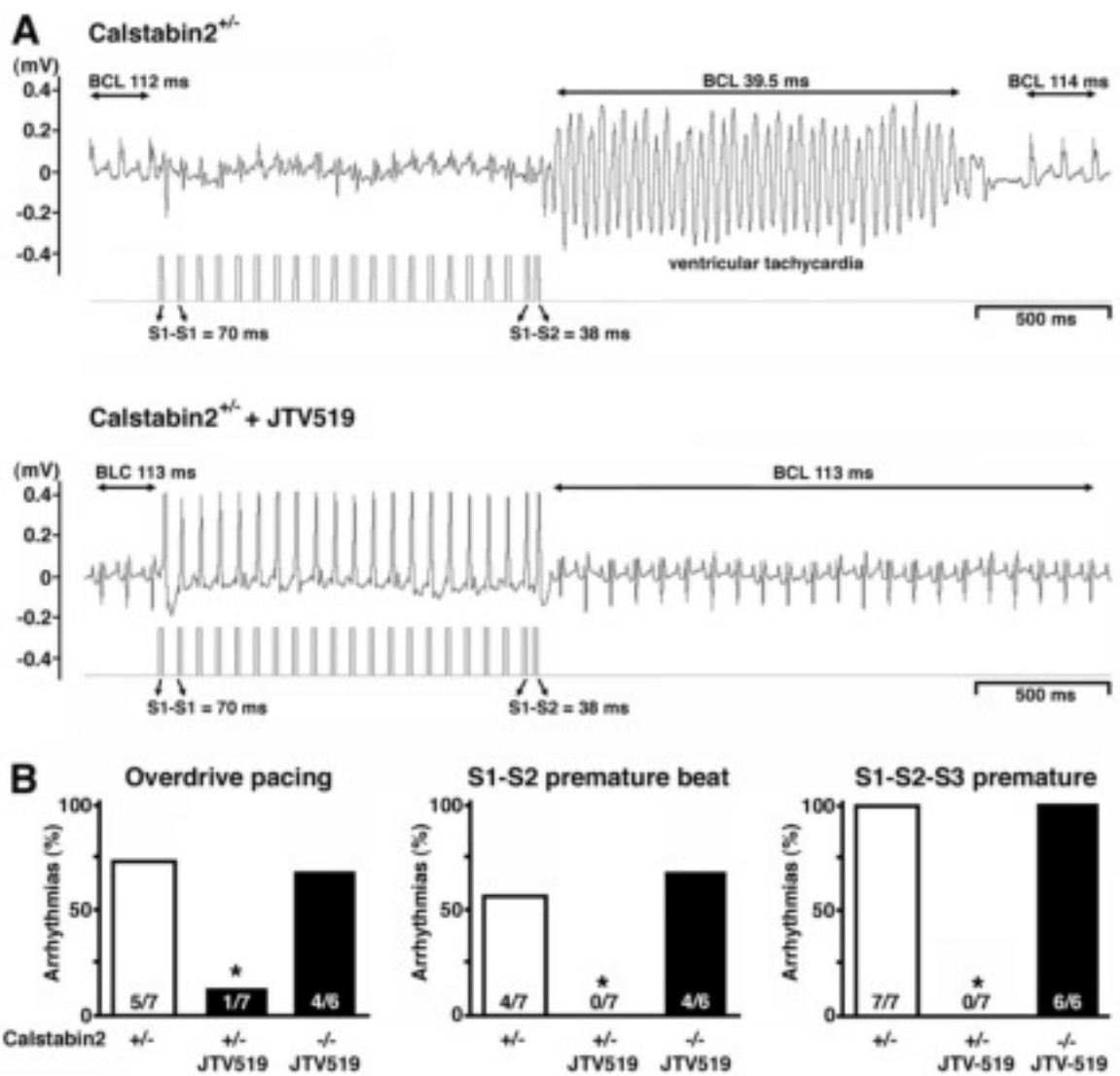

Fig. 2. Absence of antiarrhythmic actions of JTV519 in mice lacking calstabin2. (**A**) Representative ECG tracings during the programmed stimulation protocol are shown. The heart was paced epicardially at an S1-S1 interval of 70 ms, followed by a premature beat at a 38-ms S1-S2 interval. BCL, basic cycle length. (**B**) Numbers of mice with ventricular arrhythmias after overdrive pacing (left), a single S1-S2 premature beat (middle), or double S1-S2-S3 premature beats (right). *$P < 0.05$ when comparing treated and untreated calstabin2$^{+/-}$ mice.

JTV519 induced binding of calstabin2 to RyR2 (Fig. 4A). JTV519 also induced calstabin2 binding to mutant RyR2, in which Ser^{2809} was replaced by Asp (RyR2-S2809D). These channels mimic constitutively PKA-phosphorylated RyR2 channels (Fig. 4B).

The affinity of calstabin2 for PKA-phosphorylated RyR2 channels was significantly increased by addition of JTV519. The dissociation constants (K_d values) for calstabin2 binding to the channels were as follows: 148 ± 59.0 nM for RyR2-WT + PKA + PKI_{5-24} (PKA inhibitor); 1972 ± 39.9 nM for RyR2-WT + PKA; 158 ± 56.4 nM for RyR2 + PKA + JTV519 ($P < 0.05$, PKA-phosphorylated channels versus PKA-phosphorylated channels with JTV519, Fig. 4C). We obtained similar results using RyR2-S2809D channels; the K_d values for calstabin2 binding were 2123 ± 104 nM for RyR2-S2809D; and 428 ± 39 nM for RyR2-S2809D + JTV519 (Fig. 4C).

Protein kinase A phosphorylation of RyR2 activated the channel (Fig. 4, D and E), P_o = 0.01 ± 0.002 (PKA + PKI; $n = 11$) versus P_o = 0.40 ± 0.02 (PKA; $n = 12$; $P < 0.05$). Addition of calstabin2 (250 nM) to the PKA-phosphorylated RyR2-WT channels did not lower the P_o (Fig. 4E). However, addition of 1 μM JTV519 plus calstabin2 reduced the P_o back to levels comparable to those of unphosphorylated channels (Fig. 4F) (P_o = 0.002 ± 0.001; $n = 13$; $P < 0.05$).

In light of earlier studies showing that calstabin2 depletion due to PKA-hyperphosphorylation of RyR2 is associated with heart failure (*6*), an important question is why the calstabin2-deficient mice only develop arrhythmias during exercise and do not exhibit cardiac contractile dysfunction at rest. An SR Ca^{2+} leak through calstabin2-depleted RyR2 alone may not be sufficient to induce heart failure but could contribute to progression of the disease [see, e.g., (*5*)]. The effect of an isolated SR Ca^{2+} leak in an otherwise normal heart (as in calstabin2-deficient mice) could be to deplete SR Ca^{2+} stores, but this would be compensated for by increased SR Ca^{2+} uptake via SR Ca^{2+}–adenosine triphosphatase (SERCA2a). In failing hearts, the problem of increased Ca^{2+} leak is compounded by down-regulation of SERCA2a, which results in decreased SR Ca^{2+} uptake, depletes SR Ca^{2+} stores, and contributes to impaired contractility in the failing heart (*17*). Moreover, other factors, including magnesium, inhibit the opening of RyR2 channels (*18*) and are apparently able to maintain the channel in the closed state during resting nonstimulated conditions, even in the setting of calstabin2 deficiency. Only during stress, when RyR2 becomes PKA-phosphorylated, is the ability to remain closed during diastole impaired sufficiently to generate DADs and to trigger arrhythmias (*6*).

Protein kinase A phosphorylation of RyR2 does not increase the Ca^{2+} spark frequency in normal (nonfailing) cardiomyocytes under conditions that simulate diastole in the heart (cytosolic [Ca^2] of 10 or 50 nM), when normal RyR2s are tightly closed (*19*). These data are consistent with the fact that normal mice do not develop arrhythmias during exercise (*6*, *14*). Moreover, PKA-phosphorylation of RyR2 enhances RyR2 activity and increases excitation-contraction (EC) coupling gain during the early phase of EC coupling when only a small number of voltage-gated Ca^{2+} channels are open (*20*, *21*).

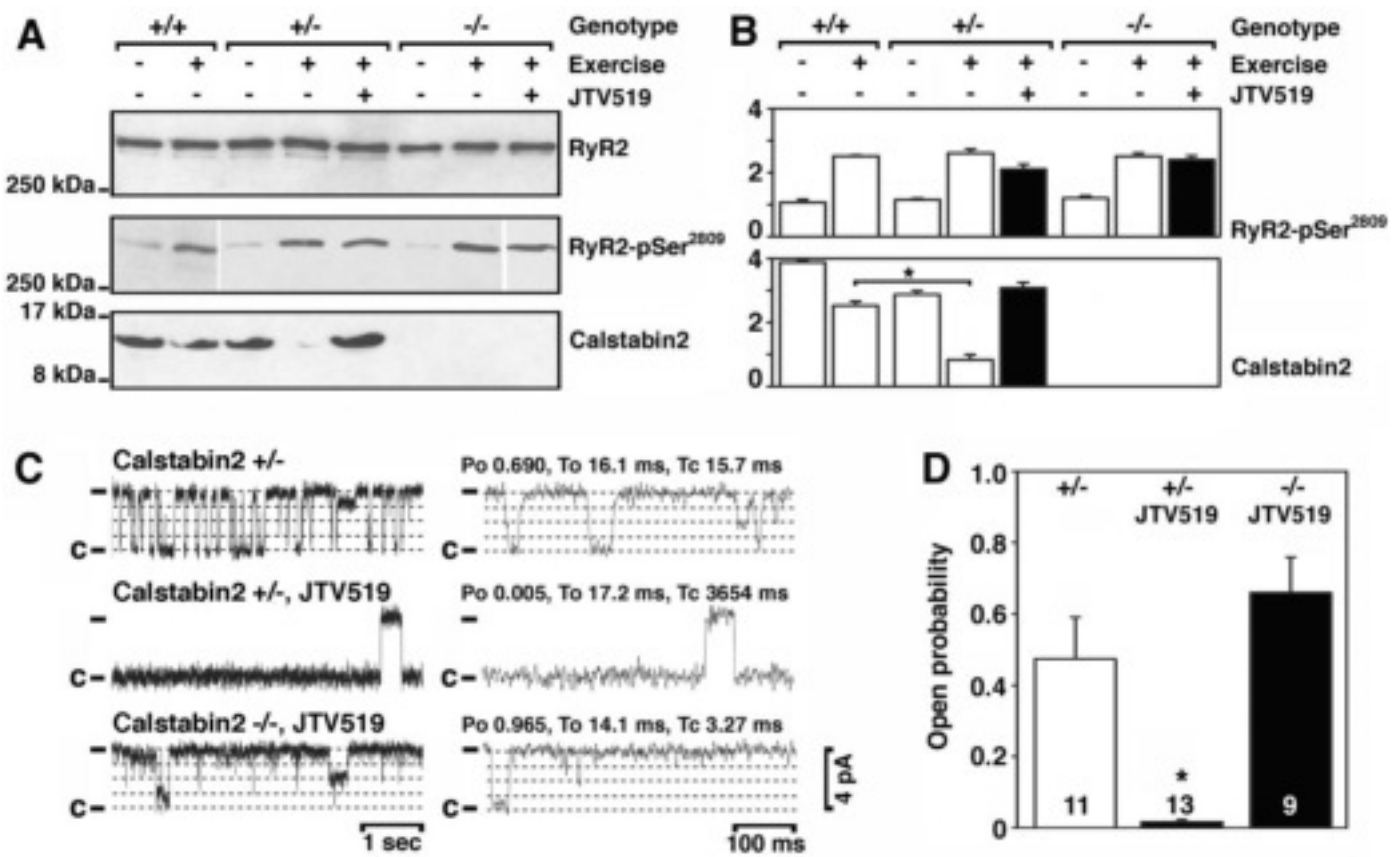

Fig. 3. Effect of JTV519 on calstabin2 affinity to RyR2 in *calstabin2*$^{+/-}$ mice after exercise. (**A**) Equivalent amounts of RyR2 were immunoprecipitated with an antibody against RyR2 [upper blot in (A)]. Representative immunoblots (A) and bar graphs (**B**) show the amount of PKA phosphorylation of RyR2 at Ser^{2809} and the amount of calstabin2 bound to RyR2 from control animals or animals after exercise immediately followed by injection with 0.5 mg/kg epinephrine. Animals were treated with JTV519 by implantable osmotic pumps (0.5 mg/kg per hour for 7 days before the exercise test). (**C**) RyR2 channels isolated from hearts after exercise testing and injection of 0.5 mg/kg epinephrine. Representative single-channel tracings of *calstabin2*$^{+/-}$ mice, both untreated and after treatment with JTV519 (0.5 mg/kg per hour), and for treated *calstabin2*$^{-/-}$ mice. Average open time (To); average closed time (Tc); closed state (c). The dotted lines indicate subconductance levels for RyR2 openings. (**D**) Average probabilities of an open channel of single RyR2 channels from *calstabin2*$^{+/-}$ or calstabin2$^{-/-}$ mice after exercise with or without JTV519 treatment. Testing was done at diastolic Ca^{2+} concentrations (150 nM). *$P < 0.05$. Numbers in the bars indicate number of single channels measured.

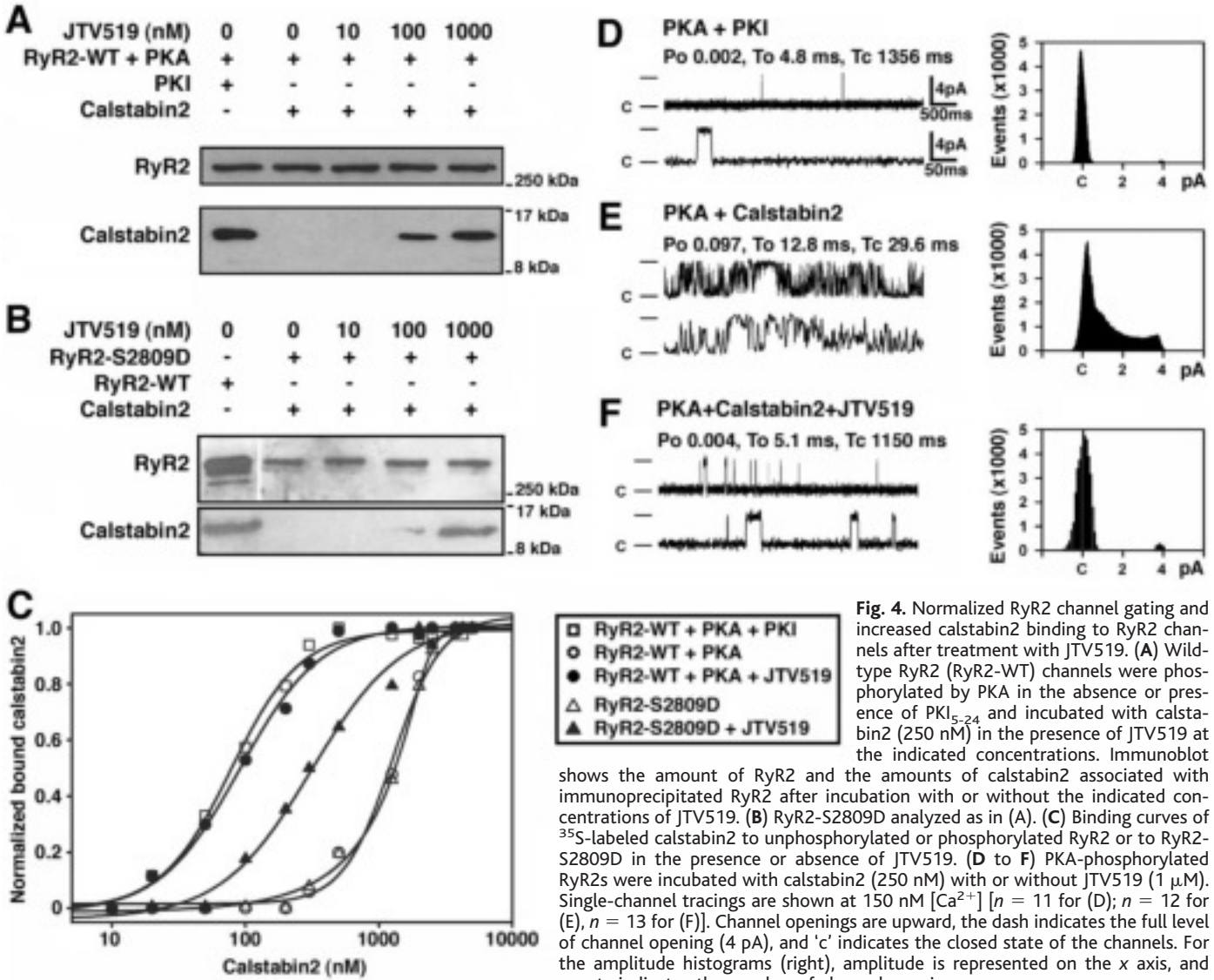

Fig. 4. Normalized RyR2 channel gating and increased calstabin2 binding to RyR2 channels after treatment with JTV519. (**A**) Wild-type RyR2 (RyR2-WT) channels were phosphorylated by PKA in the absence or presence of PKI_{5-24} and incubated with calstabin2 (250 nM) in the presence of JTV519 at the indicated concentrations. Immunoblot shows the amount of RyR2 and the amounts of calstabin2 associated with immunoprecipitated RyR2 after incubation with or without the indicated concentrations of JTV519. (**B**) RyR2-S2809D analyzed as in (A). (**C**) Binding curves of ^{35}S-labeled calstabin2 to unphosphorylated or phosphorylated RyR2 or to RyR2-S2809D in the presence or absence of JTV519. (**D** to **F**) PKA-phosphorylated RyR2s were incubated with calstabin2 (250 nM) with or without JTV519 (1 μM). Single-channel tracings are shown at 150 nM $[Ca^{2+}]$ [n = 11 for (D); n = 12 for (E), n = 13 for (F)]. Channel openings are upward, the dash indicates the full level of channel opening (4 pA), and 'c' indicates the closed state of the channels. For the amplitude histograms (right), amplitude is represented on the x axis, and events indicates the number of channel openings.

Our results show that depletion of calstabin2 from the RyR2 macromolecular complex—which is associated with increased RyR2 open probability, ventricular tachycardias, and sudden cardiac death in *calstabin2*$^{+/-}$ mice—is reversed by treatment with JTV519. We cannot exclude the possibility that calstabin2 deficiency has effects on molecules other than RyR2. However, patients with mutations in RyR2 that decrease the affinity of the channel for calstabin2 develop exercise-induced arrhythmias and sudden cardiac death (*6*), and the arrhythmogenic phenotype in mice is rescued by enhancing the affinity of calstabin2 for the channel. Thus, these data provide strong support for the concept that the leaky RyR2 channel is the important consequence of calstabin2 deficiency with regard to triggering cardiac arrhythmias. The present study identifies a molecular mechanism that may be useful for treating ventricular arrhythmias: increasing the affinity of RyR2 for calstabin2 to prevent diastolic SR calcium leaks that trigger arrhythmias. Because calstabin2 deficiency in the RyR2 macromolecular complex is a common feature in heart failure (*5*) and inherited exercise-induced ventricular arrhythmia (*6*), JTV519 may provide a specific way to treat the molecular defect in RyR2 in these disorders that triggers sudden cardiac death.

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Supporting Online Material

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References

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