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A mechanism for sudden infant death syndrome (SIDS): Stress-induced leak via ryanodine receptors

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Abstract

BACKGROUND—Sudden infant death syndrome (SIDS) is the leading cause of postneonatal mortality in the United States. Mutations in the *RyR2*-encoded cardiac ryanodine receptor cause the highly lethal catecholaminergic polymorphic ventricular tachycardia (CPVT1) in the young.

OBJECTIVE—The purpose of this study was to determine the spectrum and prevalence of *RyR2* mutations in a large cohort of SIDS cases.

METHODS—Using polymerase chain reaction, denaturing high performance liquid chromatography, and direct DNA sequencing, a targeted mutational analysis of *RyR2* was performed on genomic DNA isolated from frozen necropsy tissue on 134 unrelated cases of SIDS (57 females, 77 males; 83 white, 50 black, 1 Hispanic; average age = 2.7 months). *RyR2* mutations were engineered by site-directed mutagenesis, heterologously expressed in HEK293 cells, and functionally characterized using single-channel recordings in planar lipid bilayers.

RESULTS—Overall, two distinct and novel *RyR2* mutations were identified in two cases of SIDS. A 6-month-old black female hosted an R2267H missense mutation, and a 4-week-old white female infant harbored a S4565R mutation. Both nonconservative amino acid substitutions were absent in 400 reference alleles, involved conserved residues, and were localized to key functionally significant domains. Under conditions that simulate stress [Protein Kinase A (PKA) phosphorylation] during diastole (low activating $[Ca^{2+}]$), SIDS-associated *RyR2* mutant channels displayed a significant gain-of-function phenotype consistent with the functional effect of previously characterized CPVT-associated *RyR2* mutations.

CONCLUSIONS—Here we report a novel pathogenic mechanism for SIDS, whereby SIDS-linked *RyR2* mutations alter the response of the channels to sympathetic nervous system stimulation such that during stress the channels become “leaky” and thus potentially trigger fatal cardiac arrhythmias.

Keywords

Sudden death; Electrophysiology; Genetics; Pediatrics; Ion channels

Introduction

Sudden infant death syndrome (SIDS) is a sudden death of an infant under 1 year of age that remains unexplained after a death scene and medicolegal investigation including a complete autopsy and clinical history review.¹ Notwithstanding the achievement of the “Back to Sleep” campaign in reducing the incidence of SIDS by greater than 40% since 1992, these perplexing tragedies remain the leading cause of infant death beyond the neonatal period and the third leading cause of infant death overall in the United States, with an estimated incidence of 0.64 per 1000 live births.^{2–6} In fact, SIDS claims nearly 3000 apparently healthy infants each year, accounting for approximately 9% of the greater than 28,000 infants who die during their first year of life.⁷

Predisposing risk factors for SIDS, including prone sleeping position, sleeping on a soft surface, overheating, exposure to cigarette smoke during and after pregnancy, poor prenatal care, young maternal age with low educational levels and socioeconomic status, male sex, African American race, prematurity, and low birth weight, have been identified.^{8–12} Although many pathophysiologic theories for SIDS have been proposed, including respiratory dysfunction, cardiorespiratory instability, cardiac dysrhythmias, and inborn errors of metabolism, decisive pathogenic mechanisms triggering an infant’s sudden death remain unclear.^{13–16} However, an estimated 10% of SIDS cases may result from elusive cardiac causes such as cardiac channelopathies, including those related to a prolonged QT interval.¹⁷

Over 30 years ago, Dr. Peter Schwartz¹⁸ first proposed a hypothesis implicating the heart and the autonomic system in the involvement of SIDS, specifically long QT syndrome (LQTS). Subsequently, in 2000, Schwartz et al¹⁶ provided the first molecular proof-of-principle case report identifying a LQTS-associated mutation in a case of “near-miss” SIDS. Ackerman and colleagues^{19,20} provided molecular and functional evidence establishing LQTS-conferring cardiac channel mutations as the pathogenic basis for an estimated 5%–10% of SIDS involving Caucasian infants. Recently, Arnestad et al²¹ demonstrated a 9.5% prevalence of LQTS-associated variants among 201 Norwegian SIDS victims.

LQTS is considered a primary cardiac channelopathy involving abnormal cardiac repolarization as characterized by a prolonged QT interval on a 12-lead electrocardiogram and often manifested as syncope or sudden cardiac death (SCD) after a precipitating event such as exercise, emotion, or auditory stimuli in the setting of a structurally normal heart. On the other hand, catecholaminergic polymorphic ventricular tachycardia (CPVT) is another potentially lethal heritable arrhythmia syndrome that closely mimics LQTS and classically manifests with exertion, extreme stress, or emotion-induced syncope or sudden death in the young, including infants. Rarely, cases of resuscitated cardiac arrest and SCD during periods of sleep have been reported in individuals with CPVT.^{22,23} Like LQTS, CPVT can present with a sentinel event of SCD. Furthermore, CPVT is associated with an ostensibly normal resting 12-lead electrocardiogram and structurally normal heart, providing for its elusiveness at autopsy. Perturbations pursuant to mutations in the *RyR2*-encoded cardiac ryanodine receptor/calcium release channel represent the most common genetic subtype of CPVT.^{24–26}

In this study, in a cohort of 134 cases of SIDS, we explored the hypothesis that possible CPVT1-associated mutations pursuant to defects in the *RyR2*-encoded cardiac ryanodine receptor provide the pathogenic foundation for some cases of SIDS.

Methods

SIDS cohort

Frozen necropsy tissue from 134 cases of definite or possible SIDS (57 females, 77 males; 83 white, 50 black, 1 Hispanic; average age = 2.7 months; Table 1) was submitted for postmortem genetic testing to the Mayo Clinic Windland Smith Rice Sudden Death Genomics Laboratory between September 1997 and December 2000. Following the generally agreed-upon San Diego case definition,²⁷ SIDS was given as the official cause of death on the death certificate if autopsy, toxicology, and death scene investigation were all negative. If there was a negative autopsy, but death scene evaluation was either not performed or revealed cosleeping and the possibility of suffocation or asphyxia could not be excluded, then the cause of death was rendered undetermined or possible SIDS. Infants whose death was due to asphyxia or specific disease were excluded. The institutional review board of the Mayo Foundation approved this anonymous necropsy study. As an anonymous study, only limited medical information was available including sex, ethnicity, and age at death. Time of day, medication use, and position at death were not available.

Postmortem mutational analysis of the *RyR2*-encoded cardiac ryanodine receptor

DNA was isolated from frozen tissue using the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA). Using previously published polymerase chain reaction (PCR) primers and conditions, a targeted mutational analysis of the 28 exons of *RyR2* previously implicated in CPVT1 (3, 8, 28, 59, 14–15, 44–47, 49, 83, 87–91, 93–94, 97–105) was performed on genomic DNA using PCR, denaturing high performance liquid chromatography (DHPLC), and direct DNA sequencing as described elsewhere.²²

To be regarded as a putative SIDS-causing mutation, the genetic variant had to (1) be a nonsynonymous variant (synonymous single-nucleotide polymorphisms were excluded from consideration) (2) that involved a highly conserved residue (3) that was absent among 400 reference alleles from 100 healthy white and 100 healthy black control subjects and (4) result in a functionally altered, proarrhythmic cellular phenotype. Control genomic DNA was acquired from the Human Genetic Cell Repository sponsored by the National Institute of General Medical Sciences and the Coriell Institute for Medical Research (Camden, NJ). Mutations were annotated using the single-letter nomenclature, whereby R2267H, for example, designates a DNA alteration producing a missense mutation involving a substitution of an arginine (R) by histidine (H) at amino acid position 2267.

Ryanodine receptor mutagenesis and expression

The hRyR2-R2267H and hRyR2-S4565R mutants were generated with the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), using hRyR2 cDNA in the pCMV5 vector. The clones were sequenced to ensure that no unexpected mutations were introduced during mutagenesis. HEK293 cells grown in Minimum Essential Medium (MEM) supplemented with 10% (vol/vol) fetal bovine serum (Invitrogen, Carlsbad, CA), penicillin (100 U/mL), streptomycin (100 mg/mL), and L-glutamine (2 mmol/L) were cotransfected with 10 μ g of wild-type (WT) or mutant hRyR2 cDNA and 2.5 μ g of calstabin-2 cDNA (in 1:1 molar ratio) by Ca^{2+} -phosphate precipitation for the expression of homotetrameric channels (hRyR2-WT, hRyR2-R2267H, or hRyR2-S4565R).

Single-channel recording and data acquisition

Microsomes from HEK293 cells coexpressing calstabin-2 and RyR2-WT, RyR2-R2267H, or RyR2-S4565R mutant were prepared as described.¹ Briefly, the cell pellet was resuspended in homogenization buffer A (10 mM HEPES, pH 7.4) supplemented with a protease inhibitor cocktail on ice to allow for hypotonic lysis, followed by Teflon-glass

homogenization. After a second homogenization step with the addition of the same amount of buffer B (0.5 M sucrose, 10 mM HEPES, pH 7.4), the cell lysate was centrifuged at 1000 g for 10 minutes. Consecutive supernatants were centrifuged at 10,000 g for 15 minutes and 100,000 g for 45 minutes (Sorvall RC M120EX, rotor S100AT5). The pellet from the last centrifugation was resuspended in buffer C (0.25 mM sucrose, 10 mM HEPES, pH 7.4), frozen in liquid nitrogen, and stored at -80°C . For PKA-phosphorylated channel experiments, the microsomes were *in vitro* phosphorylated by the PKA catalytic subunit as described elsewhere.²⁸

The recombinant RyR2 channels were reconstituted by spontaneous fusion of microsomes into the planar lipid bilayer (mixture of phosphatidylethanolamine and phosphatidylserine in a 3:1 ratio; Avanti Polar Lipids, Inc., Alabaster, AL). Planar lipid bilayers were formed across a 200- μm aperture in a polysulfonate cup (Warner Instruments, Hamden, CT), which separated two bathing solutions (1 mM EGTA, 250/125 mM HEPES/Tris, 50 mM KCl, 0.5 mM CaCl_2 , pH 7.35 as *cis* solution and 53 mM $\text{Ba}(\text{OH})_2$, 50 mM KCl, 250 mM HEPES, pH 7.35 as *trans* solution). After incorporation, the activity of RyR2 channels was recorded in varying concentrations of free Ca^{2+} by consecutive additions of CaCl_2 from 20 mM stock and was calculated with the WinMaxC program (ver. 2.50; <http://www.stanford.edu/~cpatton/maxc.html>).²⁹ The identity of the channel as RyR2 was confirmed at the end of each experiment by adding ryanodine (5 mM) into the *cis* solution.

Single-channel currents were recorded at 0 mV trans-membrane potential using the Axopatch 200A patch-clamp amplifier (MDS Analytical Technologies, Toronto, Canada) in gap-free mode, filtered at 1 kHz, and digitized at 4 kHz. Data acquisition was performed using Digidata 1322A and Axoscope 9 software (MDS Analytical Technologies, Toronto, Canada). The recordings were stored on Pentium computer and analyzed using pClamp 6.0.2 (MDS Analytical Technologies, Toronto, Canada) and Origin software (ver. 6.0; OriginLab, Northampton, MA). The data in Ca^{2+} -dependence experiments were fitted with the sigmoidal three-parameters equation using SigmaPlot software (ver. 8.0, Systat Software, San Jose, CA).

Results

Molecular characterization of RyR2-encoded cardiac ryanodine receptor mutations in SIDS

Following a targeted postmortem molecular analysis using PCR, DHPLC, and direct DNA sequencing, two distinct and novel putative SIDS-causing mutations (R2267H in a 6-month-old black female and S4565R in a 4-week-old white female) in RyR2 were identified in 1/50 (2%) black infants and 1/83 (1.2%) white infants (Table 2). Figure 1 details the molecular characterization and the location of the two RyR2 missense mutations that alter highly conserved residues, were absent in 400 reference alleles (100 healthy white, 100 healthy black subjects), and localized to functionally significant domains (R2267H, near the calstabin-2-binding domain, and S4565R, the transmembrane-spanning region). In addition, these RyR2 mutation-positive SIDS cases were mutation-negative for all known LQTS susceptibility genes (data not shown). Because of the blinded nature of this study, we were unable to determine whether these RyR2 mutations represent spontaneous germ line mutations (i.e., sporadic *de novo*) or familial mutations.

Functional characterization of SIDS-associated RyR2 mutations

To determine the effects of the SIDS-associated RyR2-R2267H and RyR2-S4565R mutations on the channel function, we fused microsomes from HEK293 cells transiently coexpressing calstabin-2 and recombinant RyR2-WT (control), RyR2-R2267H, or RyR2-S4565R (Figure 2F) into a planar lipid bilayer and recorded the activity of single RyR2 channels (Figure 2A–2E).

Increased stimulation of sympathetic nervous system during exercise or certain stages of infantile sleep is associated with increased levels of intracellular cAMP through the catecholaminergic activation of β -adrenergic receptors in the heart, which leads to PKA phosphorylation of RyR2.³⁰ CPVT-associated RyR2 mutations lead to defective channel gating characterized by gain-of-function phenotype with increased open probability of the channels after PKA phosphorylation when measured under conditions that simulated diastole (low cytosolic $[Ca^{2+}]$), the resting phase of the heart cycle.³¹ These RyR2 mutations confer a leaky phenotype on the channel that leads to aberrant diastolic Ca^{2+} release from the sarcoplasmic reticulum (SR) that can initiate delayed afterdepolarizations, triggering ventricular arrhythmias and SCD.³¹

To determine whether the SIDS-linked RyR2 mutations have a similar gain-of-function leaky phenotype, we measured activity of recombinant WT and mutant channels over a range of physiological Ca^{2+} concentrations, starting at 90 nM up to full activation of the channel ($\sim 1\text{--}5\ \mu\text{M}\ Ca^{2+}$). Under basal conditions (no PKA treatment), the activity of RyR2-WT and the mutant RyR2-R2267H channels was similar at all Ca^{2+} concentrations (e.g., open probability [Po] of 0.019/0.104 for RyR2-WT versus Po of 0.022/0.098 for RyR2-R2267H at 150/700 nM Ca^{2+} ; Figure 2A, 2C), which is consistent with the clinical observation that cardiac arrhythmias in CPVT are induced typically by physical exertion or emotional stress. Interestingly, the effect of the second SIDS-associated RyR2 mutation on the channel function was different. Unlike the RyR2-R2267H mutation, the RyR2-S4565R mutant channel was more sensitive to activation by calcium (compared with RyR2-WT) under control conditions (no PKA treatment; e.g., Po of 0.58 for RyR2-S4565R versus Po of 0.104 for RyR2-WT at 700 nM Ca^{2+} ; Figure 2E, traces not shown).

To examine the functional properties of SIDS-associated RyR2 mutations under conditions simulating stress due to increased activity of the sympathetic nervous system, we recorded the activity of the channels from microsomes that have been PKA phosphorylated *in vitro*. As illustrated by the channel activity in Figure 2D, PKA-phosphorylated SIDS mutant channels exhibited significantly higher Po's (Po of 0.058/0.95 for RyR2-R2267H at 150/700 nM Ca^{2+}) over the physiologic range of Ca^{2+} concentrations in comparison with PKA-phosphorylated WT channels (Figure 2B, e.g., Po of 0.038/0.53 at 150/700 nM Ca^{2+} ; $P < .05$). A similar effect was observed for the RyR2-S4565R mutant channel, although PKA phosphorylation did not increase the activity of the channel as dramatically as for the RyR2-R2267H channel (Figure 2E). Under conditions that simulate stress (PKA phosphorylation) during diastole (low activating $[Ca^{2+}]$), SIDS-associated RyR2 mutant channels displayed a significant gain-of-function phenotype characterized by leftward shift of the half-maximal activating (stimulatory) Ca^{2+} concentration (EC_{50} of 354 ± 40 nM for PKA-phosphorylated RyR2-R2267H, $n = 3$, and EC_{50} of 555 ± 30 nM for PKA-phosphorylated RyR2-S4565R, $n = 6$, versus EC_{50} of 654 ± 57 nM for PKA-phosphorylated RyR2-WT, $n = 4$; $P < .05$; Figure 2E), consistent with the observed functional effect of previously characterized CPVT-associated RyR2 mutations.³¹

For the RyR2-R2267H mutation, this effect was not observed for channels not treated with PKA (EC_{50} of 927 ± 19 nM for RyR2-R2267H, $n = 7$, versus EC_{50} of 946 ± 23 nM for RyR2-WT, $n = 4$, not significantly different at the .05 level; Figure 2E), which supports the clinical observation that patients carrying CPVT mutations and, similarly, the infants carrying SIDS-linked RyR2 mutations do not die suddenly unless exposed to physical exercise, emotional stress, or other factors associated with increased sympathetic activity.

However, the second SIDS-associated mutation (S4565R) had a distinct effect on the channel function, which is manifested by a significant leftward shift of the Ca^{2+} -dependent activation under control conditions when the channels were not treated with PKA (EC_{50} of

662 ± 68 nM for RyR2-S4565R, n = 7, versus EC₅₀ of 946 ± 23 nM for RyR2-WT, n = 4, *P* < .05, or EC₅₀ of 927 ± 19 nM for RyR2-R2267H, n = 7, *P* < .05; Figure 2E). The sensitivity of RyR2-S4565R mutant channels to calcium under control conditions was similar to PKA-treated RyR2-WT channels (EC₅₀ of 654 ± 57 nM, n = 4, not significantly different at the .05 level; Figure 2E), which suggests that these SIDS-associated RyR2 mutant channels are more prone to become leaky and that this leak can be further enhanced during stress or increased sympathetic activity due to increased SR Ca²⁺ uptake resulting from PKA phosphorylation of phospholamban (PLB), which causes increased activity of the SR Ca²⁺-ATPase (SERCA2a).

Discussion

The present study suggests two mechanisms by which RyR2 mutations can cause diastolic SR Ca²⁺ leak that triggers fatal cardiac arrhythmias and SIDS. In one case, a mutation (R2267H) in RyR2 results in channels that exhibit normal function in nonstressed conditions but significantly increased activity in response to sympathetic stimulation as would occur during stress (e.g., hypoxia) or even possibly rapid eye movement (REM) sleep. In this case, the RyR2 channel would be prone to develop a leak during stress, and this diastolic SR Ca leak can trigger fatal cardiac arrhythmias. In the second case (S4565R), the RyR2 mutation results in channels that may be chronically leaky, even in nonstressed conditions. However, the magnitude of the leak would be enhanced during stress due to PKA phosphorylation of both RyR2 and PLB that causes both increased leak via RYR2 and increased SR Ca²⁺ loading via SERCA2a.

In addition, it is possible that the two mutations would have distinct impacts on cardiac physiology in individuals surviving beyond infancy (e.g., RyR2 mutations that cause leak only during stress might result in structurally normal hearts and CPVT in adolescence or adulthood, whereas RyR2 mutations that precipitate chronic low-level Ca²⁺ leak might be associated with a cardiomyopathy in addition to increased risk of SCD).

In this report, both SIDS infants were female and died during sleep. RyR2-associated CPVT (CPVT1) classically manifests with exertion, extreme stress, or emotion-induced syncope or sudden death predominantly expressed in young males during adolescence.^{32,33} However, in one study more than 50% of RyR2 mutation-positive, unrelated index cases were female. Moreover, nine of the 22 family members (from 10 families) experiencing an SCD were female.²² While an extremely rare phenotypic expression of CPVT, cases of resuscitated cardiac arrest and SCD during periods of sleep have been reported in individuals hosting RyR2 mutations.^{22,23}

SIDS generally occurs during periods of sleep. During sleep there can be sudden increases in sympathetic activity, which could provide the explanation for the link between CPVT and SIDS. Sympathetic activation during REM sleep was indeed one of the mechanisms first proposed in 1976 by Schwartz¹⁸ to explain how the autonomic nervous system may play a role in SIDS. Infants younger than 6 months spend 50% of their sleep in active REM sleep, compared with only 20% in adults.³⁴ During REM sleep, the dominant parasympathetic tone is interrupted by bursts of sympathetic tone, and, consequently, disruptions in cardiac rhythm may be intensified.³⁵ This physiological process may be equivalent to the surges in sympathetic nervous system activity thought to be partly responsible for initiating SCD associated with extreme emotion and heavy exertion.³⁶ Infants with an underlying cardiac channelopathy may be susceptible to such cardiac rhythmic disturbances and subsequently sudden infant death. In addition, periods of extreme emotional stress related to pain, hunger, overheating, infection, and episodes of minor upper airway obstructions from soft bedding

can be experienced during infancy.³⁷ These stressors may provide a mechanism to trigger a lethal cardiac event in RyR2 mutation–positive infants.

The finding of RyR2 mutations in 1%–2% (one of 83 white, one of 50 black) of SIDS, following this targeted analysis, adds to the growing compendium of mutations detected in genes responsible for heritable cardiac arrhythmia syndromes such as CPVT, LQTS, and Brugada syndrome. Previously, in a cohort of 93 SIDS victims (all of whom are a part of this current study), we reported LQTS mutations in three of 58 (5.2%, two SCN5A and one KCNH2) white SIDS cases and one of 34 (2.9%, one KCNQ1) black SIDS cases.^{19,20} In addition, we recently reported the finding of mutations in CAV3-encoded caveolin-3 as a novel LQTS susceptibility gene and further identified caveolin-3 mutations in three of 50 black SIDS infants.^{38,39} It is important to note that neither RyR2 mutation–positive SIDS infants hosted LQTS-associated mutations.

It is well known that 90% of SIDS occurs before 6 months of age, and that is reflected in this cohort, where 12 (9%) of the 134 infants died suddenly and unexpected at or beyond 6 months. Although the numbers are small, we have observed, interestingly, that one-third of the older SIDS cases (>6 months) were LQTS/CPVT mutation–positive compared with 4% of the infants who died before 6 months of age ($P < .004$). Recently, Arnestad et al²¹ demonstrated a 9.5% prevalence of functionally significant genetic variants in LQTS-associated genes among 201 Norwegian SIDS victims. Importantly and remarkably similar to our findings, after excluding known rare variants,^{40,41} Arnestad et al identified putative pathogenic mutations in 11 of 201 (5.5%, five KCNH2, four SCN5A, and two KCNQ1) SIDS cases. In their study, the median age was 5 months for the mutation-positive cases, compared with 3.5 months for the negative cases, but this did not reach statistical significance.

Accordingly, it appears prudent to inform parents of a SIDS victim, especially an “older” infant, that (1) postmortem cardiac channel genetic testing is available, (2) an estimated 5%–10% of SIDS may be due to LQTS and another 1%–2% may be due to CPVT, (3) perhaps as much as one-third of SIDS infants >6 months of age may be LQTS/CPVT mutation–positive, and (4) given the anonymous nature of population-based, postmortem genetic testing in SIDS, it remains unknown the extent to which LQTS/CPVT-precipitated SIDS is sporadic versus familial. As such, a recommendation for LQTS screening by electrocardiogram or CPVT screening by exercise stress testing for surviving family members of SIDS infants may be appropriate, but it should be acknowledged that compelling evidence for such clinical evaluations is lacking.

In conclusion, we have demonstrated functionally significant mutations in RyR2, the effects of which are significantly enhanced during stress and likely explain the mechanism of death in a subset of SIDS cases.

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Figure 1. Molecular characterization and localization of novel SIDS-associated RyR2 mutations. Heteroduplex analyses by DHPLC for (A) R2267H and (B) S4565R, the direct DNA sequence chromatograms for (C) R2267H and (D) S4565R, and (E) the predicted linear RyR2 channel topology (not drawn to scale) with localization of the mutations indicated by white circles. The black bar represents the calstabin-2-binding domain.

Figure 2.

Functional characterization of SIDS-associated RyR2 mutant channels. SIDS-associated mutations in RyR2 result in increased sensitivity of the channels to calcium (before or after PKA phosphorylation of the channel). **A–D**: Representative single-channel current traces of untreated and PKA phosphorylated RyR2-WT (**A**, **B**) and RyR2-R2267H (**C**, **D**) channels at two different calcium concentrations as indicated on the left (150 nM first row, 700 nM second row). At both Ca^{2+} concentrations under control conditions (no PKA treatment, *open symbols*), WT and R2267H channel activity is similar (**A** vs. **C**), however, after PKA phosphorylation (*full symbols*) open probability of RyR2-R2267H channels is significantly higher (* indicates $P < .05$) compared with PKA-treated WT channels (**D** vs. **B**). Channel activity of RyR2-S4565R under control conditions (no PKA treatment) was significantly higher compared with RyR2-WT, similar to the PKA-treated RyR2-WT. PKA phosphorylation of RyR2-S4565R further increased the sensitivity of the channels to calcium (traces not shown, see part **E** of the figure). Channel openings are shown as upward deflections; the open and closed (c) state of the channel is indicated by horizontal bars in the beginning of each trace, and corresponding amplitude histograms are on the right side of each current trace. An example of channel activity is shown at two different timescales (5 s for upper trace; 500 ms for lower trace) as indicated by dimension bars, and the P_o (open probability), T_o (average open time), and T_c (average closed time) are shown above each trace. **E**: Open probability of mutant and WT channels was measured at Ca^{2+} concentrations from 90 nM to 2 μ M (full activation). Each data point represents the open probability calculated as an average from several independent experiments shown as mean $\pm$ SE. Ca^{2+} dependences of untreated RyR2-WT (*open squares, dashed line*, $n = 4$), RyR2-R2267H (*open circles, solid line*, $n = 7$), and RyR2-S4565R (*open triangles, solid line*, $n = 7$) and PKA-phosphorylated RyR2-WT (*full squares, dotted line*, $n = 4$), RyR2-R2267H (*full circles, solid line*, $n = 3$), and RyR2-S4565R (*full triangles, dash-dotted line*, $n = 6$) were fitted by the sigmoidal equation. **F**: Untreated and PKA-phosphorylated RyR2-WT, RyR2-R2267H, and RyR2-S4565R microsomes (50 μ g) were immunoprecipitated with anti-RyR Ab. Proteins were size fractionated on 6% SDS-PAGE for RyR2 and 15% SDS-PAGE for calstabin-2. Immunoblots were developed using anti-RyR2 (5029, 1:5000 dilution), anti-

calstabin (1:2000), or a phospho-specific RyR Ab (P2809-RyR2, 1:5000 dilution) diluted in 5% milk-TBS-tween.

Table 1

Demographics of SIDS cohort

Parameter	Value
No. of SIDS cases	134
Classification:	
Definite SIDS	66
Possible SIDS	68
Gender:	
Male	77
Female	57
Age at SIDS death:	
Mean, months	2.7 ± 2.0
Range, months	0.1–12
Reported ethnicity:	
White	83
Black	50
Hispanic	1

Table 2

SIDS-associated cardiac RyR2 mutations

Sex	Ethnicity	Age	Status	Exon	Nucleotide change	Amino acid change	Location
Female	Black	6 months	Possible SIDS	45	c. 6800 G→A	p. R2267H	Calstabin 2-binding domain
Female	White	4 weeks	SIDS	94	c. 13695 C→A	p. S4565R	Transmembrane-spanning region