Supplemental Data

Characterization of recombinant RyR2-W3587A/L3591D/F3603A mutant. To determine the efficacy of the *RyR2- W3587A/L3591D/F3603A* ($RyR2^{ADA}$) mutations, we prepared initially a corresponding rabbit RyR2 mutant, which was transiently expressed in HEK293 cells and analyzed in [^{35}S]CaM binding and single channel measurements. [^{35}S]CaM binding to $RyR2^{ADA}$ was reduced at 0.4 μM Ca^{2+} as compared to wild type (WT) RyR2 and was close to background levels at 100 μM Ca^{2+} (Figure 1S). Addition of 50 nM and 1 μM CaM did not affect single channel activity of $RyR2^{ADA}$ at micromolar Ca^{2+} concentration (2 μM), whereas the open probability of WT RyR2 decreased to 70 % and 48 % of control (-CaM) in the presence of 50 nM and 1 μM CaM, respectively (Figure 2S). At 0.4 μM Ca^{2+}, 1 μM CaM inhibited both WT RyR2 and $RyR2^{ADA}$, whereas 50 nM CaM inhibited only WT RyR2 significantly. The results indicate that at resting Ca^{2+} concentration, nonphysiological concentrations of CaM are required to significantly inhibit $RyR2^{ADA}$. $RyR2^{ADA}$ bound [^{3}H]ryanodine and had a Ca^{2+}-dependence of [^{3}H]ryanodine binding identical to WT RyR2 (not shown), which showed that the mutations did not induce major changes in protein conformation.

Generation of mouse carrying RyR2-W3587A/L3591D/F3603A mutation. Figure 3S outlines the targeting strategy of generating by homologous recombination a genetically modified mouse that carried ADA mutation in *Ryr2* gene. The mouse genomic DNA encoding the RyR2 CaM binding domain (exon 75) and flanking regions were cloned into the targeting construct.

Successful targeting of the construct into mouse embryonic stem cells was verified by genomic Southern blot (Figure 3S, B), PCR and sequencing (not shown). After removing the neomycin resistant gene cassette, heterozygous ($Ryr2^{+/ADA}$) and homozygous ($Ryr2^{ADA/ADA}$) offspring were generated. Direct sequencing of RT-PCR products from total RNA of homozygous hearts confirmed replacement of the three amino acid residues W3587, L3591 and F3603 by Ala, Asp and Ala, respectively, in the CaM binding domain (Figure 3S, C). Quantitative RT-PCR analysis revealed similar RyR2 mRNA concentrations in WT and homozygous mutant hearts at 1 and 7 days. At 10 days, RyR2 transcription was significantly (30%) reduced in homozygous mutant hearts (not shown), in reasonable agreement with the B_{max} values of [^{3}H]ryanodine (Table 2).

Methods

Preparation of Mutant Mice. Mutant mice carrying the W3587A, L3591D, F3603A mutations (ADA mutations) were prepared using established methods (1). Briefly, linearized targeting vector was transfected into embryonic stem cells (TC1) by electroporation, and cells containing the recombinant gene were selected in presence of G418 and gancyclovir. ES cell clones were screened by both PCR and Southern blot, and mutations were confirmed by sequencing. The targeted ES cells were injected into the blastocysts of C57BL/6J mice in Animal Models Core Facility of the University of North Carolina at Chapel Hill. A male chimera mouse carrying mutations in *Ryr2* gene was mated with a 129/SvEv female mouse to obtain the heterozygous offspring. The neomycin resistant gene flanked by loxP sequences was removed by breeding to 129/SvEv mouse carrying transgene of Cre recombinase driven by EIIa promoter (2). Homozygous gene-targeted animals ($Ryr2^{ADA/ADA}$) were obtained by mating heterozygous mice ($Ryr2^{+/ADA}$). All experiments were done with mice having 129/SvEv

genetic background. Offspring were genotyped by PCR followed by restriction digestion with *HinfI* .

[^{3}H]Ryanodine Binding - [^{3}H]Ryanodine binding experiments were performed with crude membrane fractions from hearts. Unless otherwise indicated, membranes were incubated with 2.5 nM [^{3}H]ryanodine in 20 mM imidazole, pH 7.0, 0.15 M sucrose, 250 mM KCl, 5 mM glutathione (reduced), protease inhibitors, and indicated Ca^{2+} and CaM concentrations. Endogenous CaM was removed by addition 0.1-0.2 μM myosin light chain kinase-derived CaM binding peptide (3). Nonspecific binding was determined using a 1000-2000 fold excess of unlabeled ryanodine. After 20 h, samples were diluted with 8 volumes of ice-cold water and placed on Whatman GF/B filters preincubated with 2% polyethyleneimine in water. Filters were washed with three 5 ml ice-cold 100 mM KCl, 1 mM KPipes, pH 7.0 solution. The radioactivity remaining with the filters was determined by liquid scintillation counting to obtain bound [^{3}H]ryanodine.

B_{max} values of [^{3}H]ryanodine binding were determined by incubating membranes for 4 h at 24^{o}C with a saturating concentration of [^{3}H]ryanodine (30 nM) in 20 mM imidazole, pH 7.0, 0.6 M KCl, 0.15 M sucrose, 20 μM leupeptin, 200 μM Pefabloc, and 100 μM Ca^{2+}. Specific binding was determined as described above.

Single Channel Recordings - Single channel measurements of WT and mutant RyR2s were performed in planar lipid bilayers containing phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine in the ratio of 5:3:2 (25 mg of total phospholipid per ml of n-decane) (4). Crude cardiac membrane fractions containing WT and mutant RyR2s were pretreated for 30 min with 1 μM myosin light chain kinase-derived CaM binding peptide to dissociate endogenous CaM. Final peptide concentration was 10 nM following the addition of

membranes to the cis (cytosolic) chamber of the bilayer apparatus. A strong dependence of single channel activities on cis Ca^{2+} concentration indicated that the large cytosolic "foot" region faced the cis chamber of the bilayers. The trans (SR lumenal) side of the bilayer was defined as ground. Unless otherwise indicated, measurements were made with symmetrical 0.25 M CsCl, 20 mM CsHEPES, pH 7.4 with indicated concentrations of Ca^{2+}. Exogenous CaM was added to the cis solution. Electrical signals were filtered at 2 kHz, digitized at 10 kHz and analyzed as described (4). P_o values in multichannel recordings were calculated using the equation $P_o = \sum iP_{o,i}/N$, where N is the total number of channels and $P_{o,i}$ is channel open probability of the ith channel.

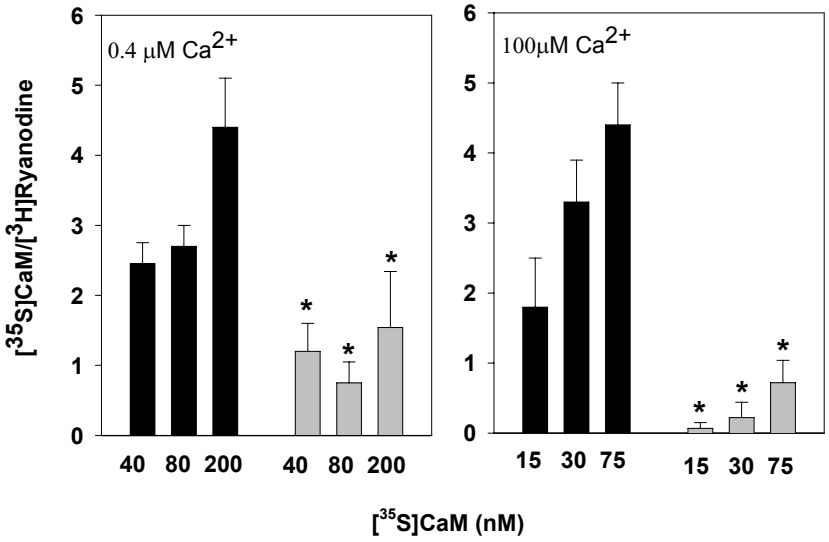

Figure 1S. [^{35}S]CaM binding to WT-RyR2 and RyR2-W3587A/L3591D/F3603A expressed in HEK293 cells. Membrane fractions prepared from HEK293 cells expressing WT-RyR2 (black bars) and RyR2-W3587A/L3591D/ F3603A (grey bars) were incubated for 2.5 h at 24^{o}C with 15-200 nM [^{35}S]CaM in 20 mM imidazole, pH 7.0, 0.15 M sucrose, 150 mM KCl, 0.125 mg/ml BSA, 5 mM glutathione (reduced), 20 μM leupeptin, 200 μM Pefabloc, and either 0.4 μM free Ca^{2+} or 100 μM free Ca^{2+}. Specific [^{35}S]CaM binding was determined by a sedimentation assay as described **(3).** The ratios of [^{35}S]CaM binding to maximal [^{3}H]ryanodine binding were obtained, taking into account that there is one high-affinity [^{3}H]ryanodine binding site per RyR2 tetramer. Data are the mean ± S.E. of 4-7 experiments. *p<0.05 compared to respective WT values, as determined with Student's t test.

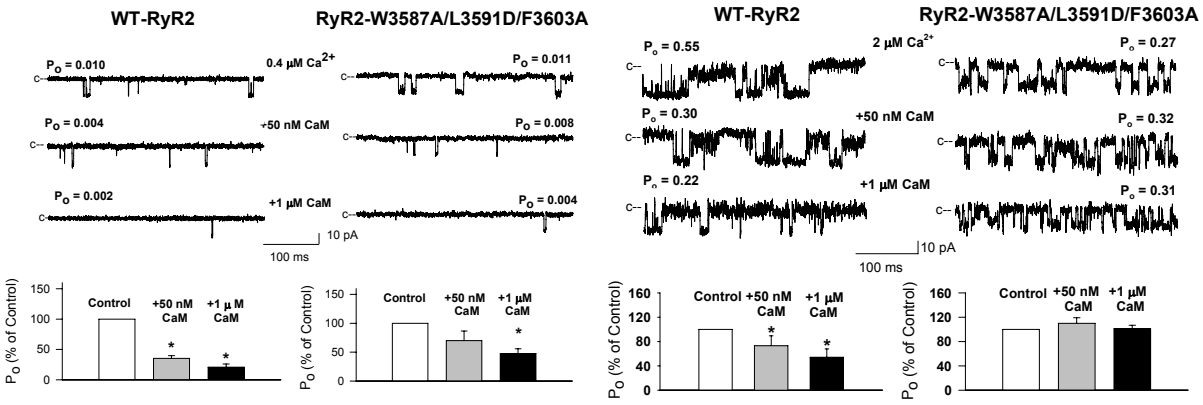

Figure 2S. CaM inhibition of WT-RyR2 and RyR2-W3587A/L3591D/F3603A expressed in HEK293 cells. Crude membrane fractions isolated from HEK 293 cells that expressed WT RyR2 or RyR2-W3587A/L3591D/ F3603A were fused with a lipid bilayer (4). Representative single channel recordings of WT-RyR2 and RyR2-W3587A/L3591D/ F3603A are shown. Single channel currents were recorded at -20 mV in symmetric 0.25 M KCl media in the presence of 0.4 μM (A) or 2 μM (B) free cis (cytosolic) Ca^{2+} before (top trace) and after the addition of 50 nM (middle trace) and 1 μM CaM (bottom trace). (Bottom panels) Averaged normalized channel open probabilities ± S.E. of 4-8 channel recordings. *p<0.05 compared to control (-CaM), as determined with Student's t test.

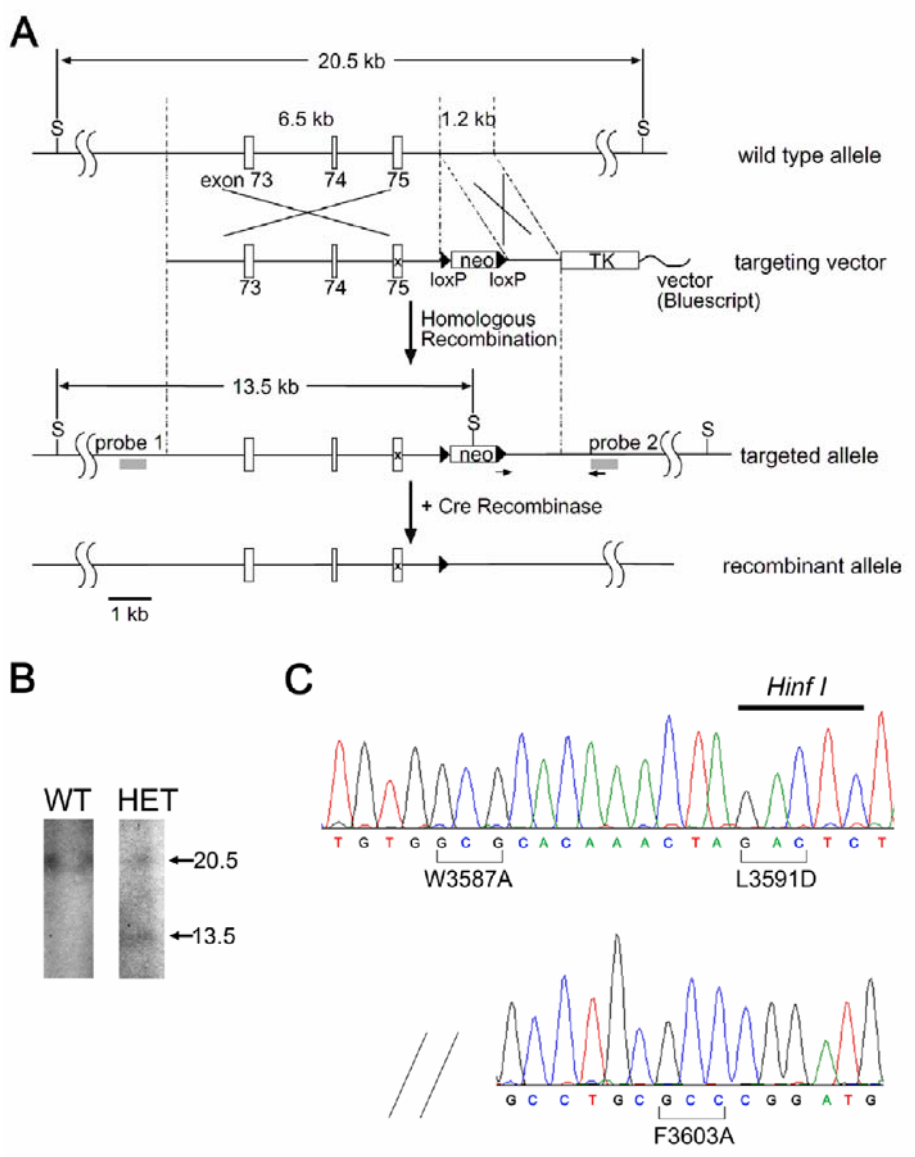

Figure 3S. Generation of mice with mutations in CaM binding site of RyR2. (A) Schematic representation of the mouse *Ryr2* genome and targeting construct. S, neo, and TK denote *SphI* enzyme site, neomycin resistant gene, and thymidine kinase gene, respectively. Arrows and 'x' indicate the position of primers for screening and mutation site, respectively. (B) Southern blot analysis of genomic DNA. A 5' probe (Probe 1) identifies 20.5 kb *SphI* fragment in wild-type allele and 13.5 kb fragment in targeted allele. Hybridization with 3' probe (Probe 2) showed consistent results (not shown). (C) Sequence analysis of RT-PCR. cDNA encoding CaM binding site of RyR2 was amplified from total RNA of homozygous mouse heart and sequenced. Three mutations (W3587A, L3591D and F3603A) were confirmed. A *HinfI* site created by the L3591D mutation (GACTC) was used for screening the mutant allele.

References

1. Bronson, S.K., Plaehn, E.G., Kluckman, K.D., Hagaman, J.R., Maeda, N., and Smithies, O. 1996. Single-copy transgenic mice with chosen-site integration. *Proc. Natl. Acad. Sci. USA* **93**:9067-9072.

2. Lakso, M., Pichel, J.G., Gorman, J.R., Sauer, B., Okamoto, Y., Lee, E., Alt, F.W., and Westphal, H. 1996. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. *Proc. Natl. Acad. Sci. USA* **93**:5860-5865.

3. Yamaguchi, N., Xu, L., Pasek, D.A., Evans, K.E., Chen, S.R.W., and Meissner, G. 2005. Calmodulin regulation and identification of calmodulin binding region of type-3 ryanodine receptor calcium release channel. *Biochemistry* **44**:15074-15081

4. Xu, L., Wang, Y., Gillespie, D,. and Meissner, G. 2006. Two rings of negative charges in the cytosolic vestibule of type-1 ryanodine receptor modulate ion fluxes. *Biophys. J.* **90**:443-453.