Supplemental Data

Phosphodiesterase 4D Deficiency in the Ryanodine-Receptor Complex Promotes Heart Failure and Arrhythmias

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Supplemental Experimental Procedures

RyR2-S2808A Knockin Mice

Mouse genomic λ-phage clones for segments of the murine ortholog of the human *RyR2* gene were isolated from a 129/SvEvTacfBR genomic library (Stratagene, La Jolla, CA). A 5.4kb Eco RI fragment containing exons 53 to 55 and the flanking intronic regions was isolated using a 250 bp ^{32}P-labeled cDNA probe containing serine (S) 2808. The isolated 5.4kb fragment was subcloned into the Eco RI site of pBluescriptSK, and S2808 was mutated to alanine (A) using a Chameleon Mutagenesis Kit (Stratagene, La Jolla CA). In addition to the S2808A mutation, an extra FSP I restriction site was added to exon 55. The 5.4kb Eco RI fragment was then excised and cloned into the Eco RI site of pACN vector. The pACN plasmid was a backbone vector containing a cassette (ACN) with genes for neomycin resistance, Cre recombinase and a testes-specific promoter (tACE), flanked by loxP sites. The promoter tACE initiates expression of Cre recombinase only during spermatogenesis, resulting in excision of the ACN cassette. The 3' targeting arm, consisting of the 2463 bps upstream of the Eco RI site, was obtained by PCR of genomic mouse DNA. After adding Sal I sites to both ends of this 2.4 kb fragment, it was cloned into the Sal I site of the pACN vector containing the mutated 5.4kb segment. The Kpn I linearized targeting vector was electroporated into MM13 mouse embryonic stem (ES) cells using established protocols. Gene-targeted ES cells were screened by Southern analysis using both 5' and 3' external probes to confirm homologous recombination, and injected into C57Bl6 blastocysts. Founder mice were backcrossed to C57Bl6 mice. Germline offspring were identified by brown coat color and further verified by Southern blot analysis. Heterozygous males and females were intercrossed to obtain homozygous offspring.

Transthoracic Echocardiography and In Vivo Hemodynamic Analysis in Mice

All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Columbia University and according to NIH guidelines. For transthoracic 2-D echocardiography, mice were anesthetized with 1.0-1.5% isoflurane in O_2 and placed on a 37°C heating pad. Hearts were visualized parasternally along the short axis to obtain 2-D images and M-mode tracings of the anterior wall, left ventricular cavity, and posterior wall. Left ventricular dimensions and function were measured in triplicate from different cardiac cycles for the number of animals indicated.

Hemodynamic measurements were performed on *PDE4D*$^{-/-}$, *PDE4D*$^{+/-}$ and age-and litter-matched wild-type (WT) mice (3 to 15 months) anesthetized with 1.5% isoflurane using a 1.4 F micromanometer-conductance catheter (SPR-839, Millar Instruments) via the right carotid artery. Pressure-volume analysis was performed using hemodynamic analysis software (EMKA Technologies, VA) as described (van Rooij et al., 2004).

FRET-PKA Assay

Primary cultures of adult murine ventricular cardiac myocytes from wild-type and age-and litter-matched *PDE4D*$^{-/-}$ hearts were isolated using a modified Langendorff perfusion protocol with Ca^{2+} free Tyrode solution followed by collagenase digestion (Type II, Worthington). Cardiomyocytes were infected with recombinant adenoviruses expressing CFP attached to the PKA regulatory subunit (RII-CFP) and YFP attached to the PKA catalytic subunit (C-YFP) as described previously (Warrier et al., 2005). Simultaneous infection of mouse cardiomyocytes occurred

at a multiplicity of infection of 50 to 100 for each virus. Cells expressing approximately equal amounts of CFP and YFP as evidenced by fluorescence at 48-72 hrs, were used for intracellular cAMP imaging by FRET. Imaging was performed with an inverted microscope (Olympus IX70) equipped with a 40X water immersion objective (1.3 NA, Olympus) and a CCD camera (Hamamatsu, Orca ER) as described previously (Warrier et al., 2005). Fluorescence images were acquired using 2x2 binning and analyzed using Simple PCI imaging software (Compix Inc.) and changes in cAMP concentrations at the Z-line containing RyR2 complexes were defined as the relative changes in the intensity of CFP and YFP measured at the Z-lines within a region of interest. Isoproterenol bitartrate (Iso; Sigma RBI) was prepared as stock solution and applied by rapid perfusion.

Immunoprecipitation and Immunoblot Analysis

RyR2 channels were immunoprecipitated from 100 μg of human or murine cardiac homogenates using anti-RyR antibody (Jayaraman et al., 1992) in 0.5 ml of RIPA buffer (50 mM Tris-HCl buffer), pH 7.4, 0.9% NaCl, 5.0 mM NaF, 1.0 mM Na_3VO_4, 0.25% Triton-X100, and protease inhibitor mix (Roche) overnight at 4°C. The use of human tissues was approved by the Institutional Review Board of Columbia-Presbyterian Medical Center. Normal and failing human heart tissues were obtained as previously described from patients undergoing cardiac transplant (Marx et al., 2000). Immunoprecipitates were separated by SDS-PAGE and the proteins were transferred onto nitrocellulose membranes overnight (Semi-Dry transfer blot, Bio-Rad, USA). Immunoblots were developed with an enhanced chemiluminescence system using primary antibodies against RyR (5029; 1:3,000) (Jayaraman et al., 1992), PDE splice-variant 4D3 (1:1,000) (Reiken et al., 2003b) calstabin2 (1:1,000) (Wehrens et al., 2003), PKA catalytic subunit (1:1,000), PP1 (1:1,000) and PP2A (1:1,000) (Transduction Labs, Lexington, KY) (Marx et al., 2000). PKA phosphorylation of RyR2 was quantified using phospho-epitope specific RyR2-pSer^{2808} antibody (1:5,000) (Reiken et al., 2003c). Results were confirmed using a PKA back-phosphorylation assay as previously described (Reiken et al., 2003c). CaMKII phosphorylation of RyR2 was quantified using phospho-epitope specific antibody RyR2-pSer^{2814} (1:5,000) as described (Wehrens et al., 2004). Band densities were quantified using Quantity One software (Biorad, Hercules, CA) (Reiken et al., 2003a). Data presented represent ≥4 individual experiments.

Back Phosphorylation of PDE4D3

PDE4D3 was immunoprecipitated from 100 μg of human cardiac homogenates with anti-PDE4D3 antibody (5 μg/ml) in 0.5 ml of RIPA buffer overnight at 4°C. Samples were incubated with Protein A sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) at 4°C for 1 hour, beads were washed three times with 1x kinase buffer (8 mM $MgCl_2$, 10 mM EGTA, and 50 mM Tris/piperazine-N,N'-bis(2ethanesulfonic acid), pH 6.8). After resuspending the beads in 10 μl of 1.5 x kinase buffer containing PKA catalytic subunit (5 units, Sigma, St. Louis, MO), back phosphorylation of the immunoprecipitated PDE4D3 was initiated with 5 μl of 100 μM Mg-ATP containing 10% [γ-^{32}P]-ATP (NEN Life Sciences, Boston, MA). The reaction was terminated after 10 min at RT with 5 μl of stop solution (4% SDS and 0.25 M DTT). Samples were heated to 95ºC, size fractionated on 8% PAGE, and PDE4D3 radioactivity was quantified using a Molecular Dynamics Phosphorimager and ImageQuant software (Ammersham Pharmacia).

β-Adrenergic-Receptor Measurements

Aliquots of cardiac membrane preparations from 5 wild-type (WT) and 5 *PDE4D*$^{-/-}$ knockout mice were incubated for 2 hours in 0.5 mM Tris-HCl buffer, pH 7.4, containing increasing concentrations of [^{125}I]-(-)-cyanopindolol ([^{125}I]-CYP) before the reactions were filtered using GF/C microfiber filters from Whatman. The filters were washed three times with 3 ml of binding buffer, dried, and bound radioligand was measured in a γ-radiation counter. [^{125}I]-CYP binding was determined in the presence and absence of 1 μM alprenolol to distinguish between specific and nonspecific (residual binding in the presence of 1 μM alprenolol) binding.

PKA Phosphorylation of Cardiac Ryanodine Receptors

RyR2 was immunoprecipitated from 250 μg of mouse heart homogenate. PKA phosphorylation of RyR2 was initiated with 5 μl of 100 μM Mg-ATP (for autoradiography, the Mg-ATP contained 10% [^{32}P]-γATP (NEN Life Sciences, Boston, MA) in kinase buffer (8 μM $MgCl_2$, 10 mM EGTA, and 50 mM Tris/piperazine-N,N'-bis(2ethanesulfonic acid), pH 6.8). The reaction was terminated after 8 min at room temperature with 5 μl of stop solution (4% SDS and 0.25 M DTT). Samples were heated to 95 °C and size-fractionated on 6% SDS-PAGE.

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