

Muscarinic Stimulation Facilitates SR Ca Release by Modulating RyR2 Phosphorylation Through PKG and CaMKII

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Online Supplement

Methods

Mouse Model

Wild-type (WT) and RyR2 knock-in (RyR2-S2808A)¹ 3-6 month old male mice were used in this study. Ventricular cells were isolated using Liberase TH Research Grade enzyme (Roche) as previously described.^{2,3} For Ca imaging, cells were loaded with the Ca-sensitive indicators Fluo-4 FF AM (10 $\mu\text{mol/L}$) for 25 minutes followed by an additional 25 minutes for de-esterification. Loaded cells were excited with the 488 nm line of an argon laser and emission was collected at 500-600 nm. The fluorescence was recorded in the line scan mode of an Olympus Fluoview 1000 confocal microscope. The external, Tyrode solution contained (mmol/L): 140 NaCl, 5.4 KCl, 1 or 2.5 CaCl_2 , 0.5 MgCl_2 , 10 Hepes and 5.6 glucose (pH 7.3). Myocytes were paced at 0.5 Hz using extracellular platinum electrodes. Experiments were performed within 4 hours after myocyte isolation.

Canine Model

Out-bred hound type dogs were obtained consistent with NIH guidance. Young adult dogs (1-3 years, 15-30 kg) of either sex were used, and either had heart failure induced or served as controls. Heart failure was induced by a minimum of 16 weeks of right ventricular tachypacing.⁴⁻⁶ Serial echocardiograms were used to assess left ventricular function and cardiac chamber dimensions. This 16-week heart failure model emulates all aspects of human heart failure including atrial and ventricular dilatation, impaired systolic function, myocyte hypertrophy, elevated catecholamines, and decreased functional capacity assessed by six-minute walk test.

Ventricular myocytes were prepared following established procedures.⁴⁻⁶ To monitor intracellular Ca and reactive oxygen species (ROS), Ca- and ROS-sensitive indicators Rhod-4 AM (10 $\mu\text{mol/L}$) and CM-H₂DCFDA AM (10 $\mu\text{mol/L}$) were used, respectively. Similar to mouse myocytes, cells were loaded with the dyes for 25 minutes followed by an additional 25 minutes for de-esterification. Rhod-4 was excited by 543 nm laser and emission was collected at 590-690 nm whereas CM-H₂DCFDA was excited by 488 nm line of an argon laser and signal was collected at 500–560 nm. Myocytes were paced at 0.3, 0.5, 1 or 2 Hz using extracellular platinum electrodes in normal 2 mmol/L Ca Tyrode solution. Experiments were performed within 7 hours after myocyte isolation.

Biochemistry Assays

Cardiac cells were isolated, frozen in liquid nitrogen and kept in -80 °C until use. Protein concentrations were determined by Bradford assay. Cardiac homogenates (30-50 μg) were subjected to 4% to 15% SDS-PAGE, and blotted onto nitrocellulose membranes. Phosphorylation status of proteins was detected using phospho-specific antibodies: RyR2 Ser-2808 (Badrilla, A010-30P), Ser-2814 (Badrilla, A010-31P) and Ser-2030 (antibody was a generous gift from Dr. Héctor Valdivia) were normalized to total RyR2 (Thermo Scientific, MA3-916); GAPDH (Fitzgerald, 10R-G109A) was used as loading control. The blots were developed with Super Signal West Pico (PIERCE) and quantified using ImageJ software.

Reference

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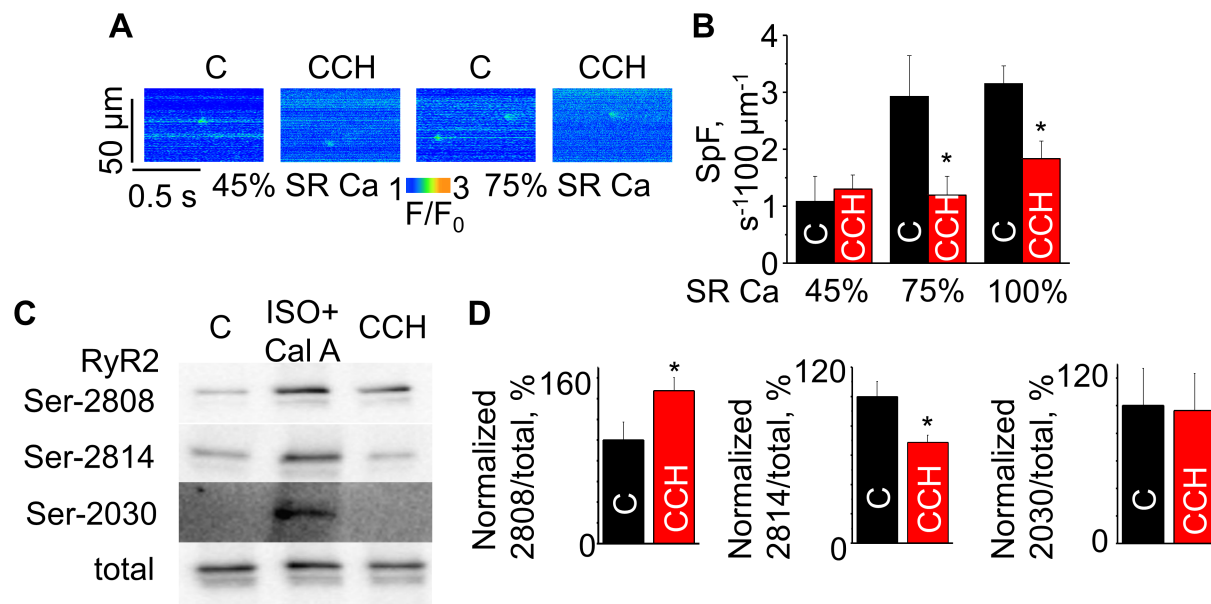


Fig. S1 Effects of CCH on Ca handling and RyR2 phosphorylation in non-failing canine ventricular myocytes. (A) Confocal images of Ca sparks recorded at various levels of the SR Ca content with or without 10 $\mu\text{mol/L}$ CCH. **(B)** Pooled data for the frequency of Ca sparks (SpF) observed at various levels of the SR Ca contents ($\pm\text{SEM}$, $n=6-10$). * $P<0.05$ vs control (C). Immunoblots **(C)** and summary data **(D)** for the effect of CCH on phosphorylation of RyR2 ($\pm\text{SEM}$, $n=3$). * $P<0.05$, paired t-test. Cal A (Calyculin A) + ISO produces maximum phosphorylation. SR Ca content was obtained by application of 20 mmol/L caffeine.

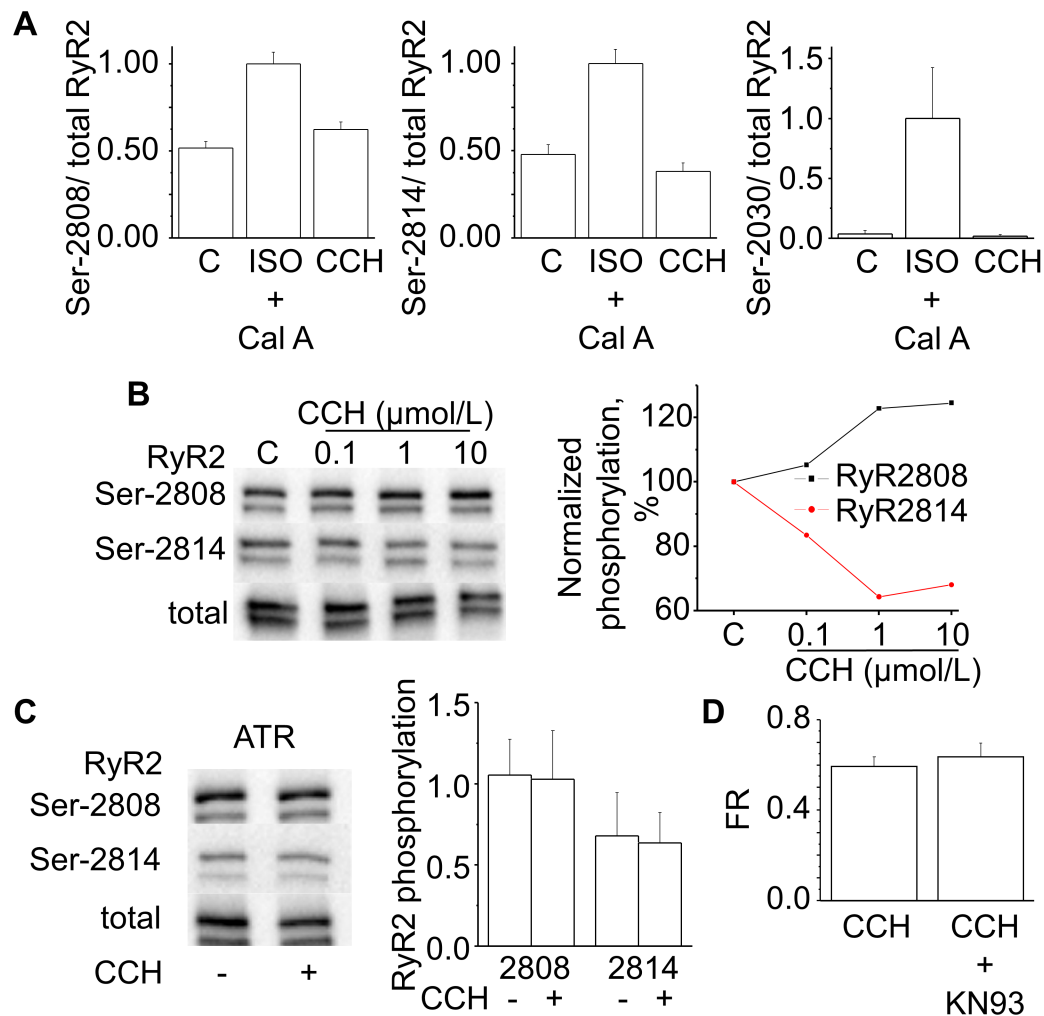


Fig. S2 CCH causes reciprocal RyR2 phosphorylation on Ser-2808 and dephosphorylation on Ser-2814. (A) Ser-2808 baseline phosphorylation compared to ISO+Cal A as maximum phosphorylation. (\pm SEM, $n=4-18$). (B) Immunoblot and densitometric analysis of CCH dose response obtained in mouse ventricular myocytes. (C) Representative immunoblots (left) and pooled data (right) obtained in mouse ventricular myocytes in the presence of the muscarinic receptor antagonist, atropine (ATR) with or without CCH (\pm SEM, $n=4$). (D) Effects of CaMKII inhibition by KN-93 on fractional release (FR) in the presence of CCH (\pm SEM, $n=14$).

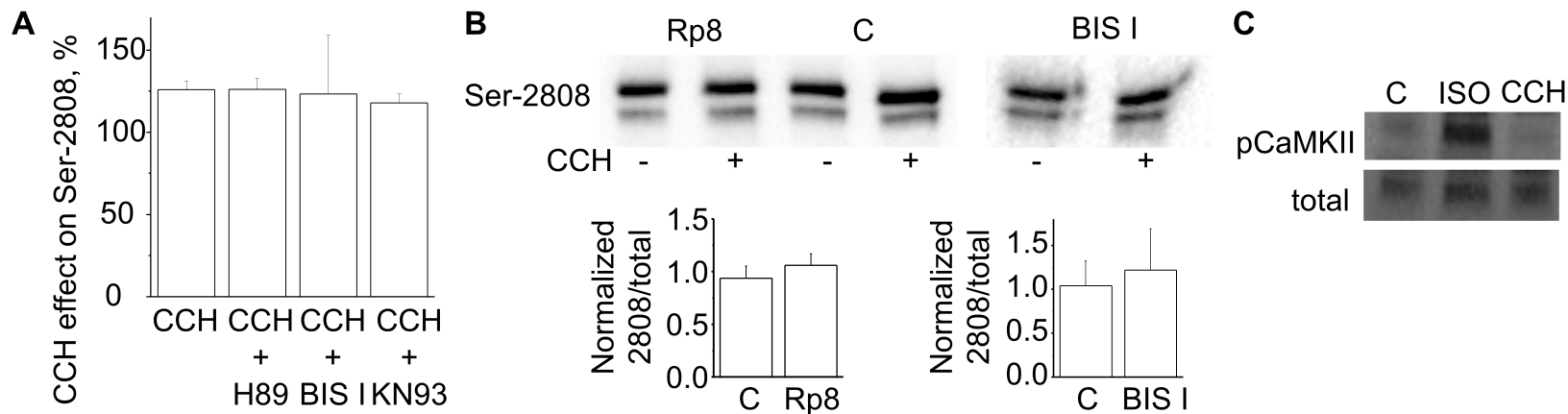


Fig. S3 Ser-2808 baseline phosphorylation and kinases inhibitions. **(A)** Effects of kinase inhibitions by H89 (PKA), BIS I (PKC) and KN-93 (CaMKII) on Ser-2808 in the presence of CCH (\pm SEM, n=2-7). **(B)** Representative immunoblots and pooled data obtained in mouse ventricular myocytes for Ser-2808 baseline in the presence of PKG inhibitor (Rp8) and PKC inhibitor (BIS I) (\pm SEM, n=3-8). **(C)** Immunoblot for CaMKII activity.