SUPPLEMENTAL MATERIAL

for

The novel FRET-based sensor Camui provides new insight into mechanisms of CaMKII activation in intact cardiomyocytes

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METHODS

Construction of Adenoviral Vectors Encoding Biosensors

The Camui construct[1] was incorporated in adenoviruses using the AdEasy™ adenoviral vector system (Qbiogene, Inc., Carlsbad, CA) to ensure high infection efficiency in the terminally differentiated adult ventricular myocytes. Mutant variants of Camui (T286A and MM280/281VV) were generated using the commercially available QuickChange site directed mutagenesis kit (Stratagene), and likewise incorporated into adenovirus.

HEK293 Cell Transfection

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) with 5% fetal bovine serum and penicillin/ streptomycin for 24 h and then transiently transfected with expression plasmids encoding Camui using a mammalian transfection kit (Stratagene). Cells were cultured for an additional 36 hours post transfection. Camui expression was checked by fluorescence microscopy prior to experiments.

In Vitro Fluorescence and CaMKII activity assays

Fluorescence measurements were performed using a MS SpectraMax plate reader spectrophotometer (Molecular Devices). Excitation and emission slits were set at 4 nm. Excitation wavelength of 440 nm was used, and dual photon counting emission detectors were set at 477 nm (F_{CFP}) and 527 nm (F_{YFP}), respecttively. The cytosolic fraction of the transfected HEK cells was diluted in Ca^{2+}-free buffer containing 50 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, and protease inhibitors. Camui fluorescence was measured in the presence of 10 μM CaM and 200 μM Ca^{2+}. For some experiments, 1 mM EGTA was used to chelate Ca^{2+}. Autonomous CaMKII activity was measured in the presence of 1 mM EGTA, 100 μM ATP and/or 1 μM H_2O_2. Incubation time was five minutes to allow achievement of steady state. Measurements were made in black/clear bottom tissue culture plates (Costar) at 37°C. CaMKII kinase activity was confirmed by measuring incorporation of ^{32}P-ATP into an artificial substrate, syntide-2, as previously described.[2] Assays were performed on either purified kinase or on HEK cell lysates expressing Camui, as described in the Results section. Incubation times were five minutes as before, to achieve steady state. Data were normalized against background measurements lacking syntide-2.

Myocyte Isolation and Adenoviral Infection

All protocols involving animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the University of California, Davis Institutional Animal Care and Use Committee. Adult rabbit ventricular myocytes were isolated as previously described.[3] Myocytes were seeded on laminin-coated coverslips in serum-free PC-1 medium (Lonza) supplemented with penicillin/streptomycin. Myocytes were infected for 2 hours at multiplicity of infection of 10–100 with adenovirus encoding Camui, followed by replacement with fresh medium. Infected cells were kept in culture for 36 hours with one final replacement of fresh medium 1 hr before experiments.

Confocal Microscopy Imaging

Cover slips were mounted on the stage of an inverted microscope (Zeiss, LSM5 Pascal) equipped with a 40× 1.4 NA water immersion objective lens. Argon laser excitation wavelengths were 458 nm for CFP and 514 nm for YFP. CFP emission fluorescence was measured by confocal microscopy at 485 ± 15 nm, while YFP emitted fluorescence was measured at ≥535 nm. For some experiments, cells were field stimulated at 0.25, 0.5, and 1 Hz. Some cells were treated with 1 μM angiotensin II, isoproterenol, phenylephrine, or endothelin-1. All myocyte experiments were performed in Tyrode solution containing 1 mM Ca^{2+} unless otherwise noted. For each experimental condition, a minimum of 30 cells (3 rabbits, 10 cells from each) was analyzed. Image-J software was used for image analysis.

Statistics

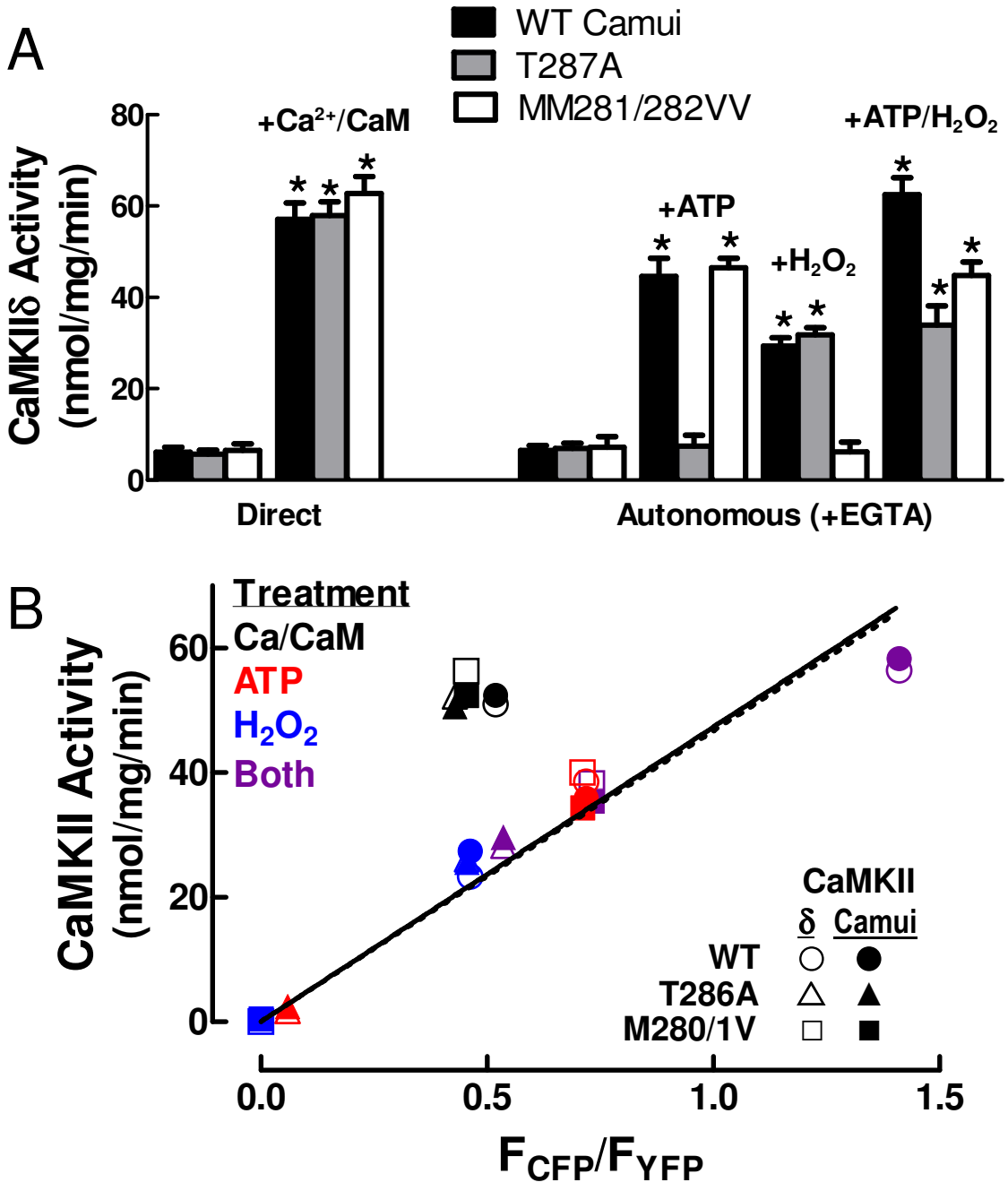
Pooled data are represented as the means ± S.E. Statistical comparisons were made using repeated two-way analysis of variance and paired Student's t test where applicable. $p < 0.05$ was considered significant.

REFERENCES

1. Kwok S, Lee C, Sánchez SA, Hazlett TL, Gratton E, Hayashi Y. Genetically encoded probe for fluorescence lifetime imaging of CaMKII activity. *Biochem Biophys Res Commun*. 2008:519-25.
2. Erickson JR, Joiner ML, Guan X, Kutschke W, Yang J, Oddis CV, Bartlett RK, Lowe JS, O'Donnell SE, Aykin-Burns N, Zimmerman MC, Zimmerman K, Ham AJ, Weiss RM, Spitz DR, Shea MA, Colbran RJ, Mohler PJ, Anderson ME. A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. *Cell*. 2008:462-74.
3. Bassani JW, Bassani RA, Bers DM. Calibration of indo-1 and resting intracellular $[Ca]_i$ in intact rabbit cardiac myocytes. *Biophys J*. 1995:1453-60.

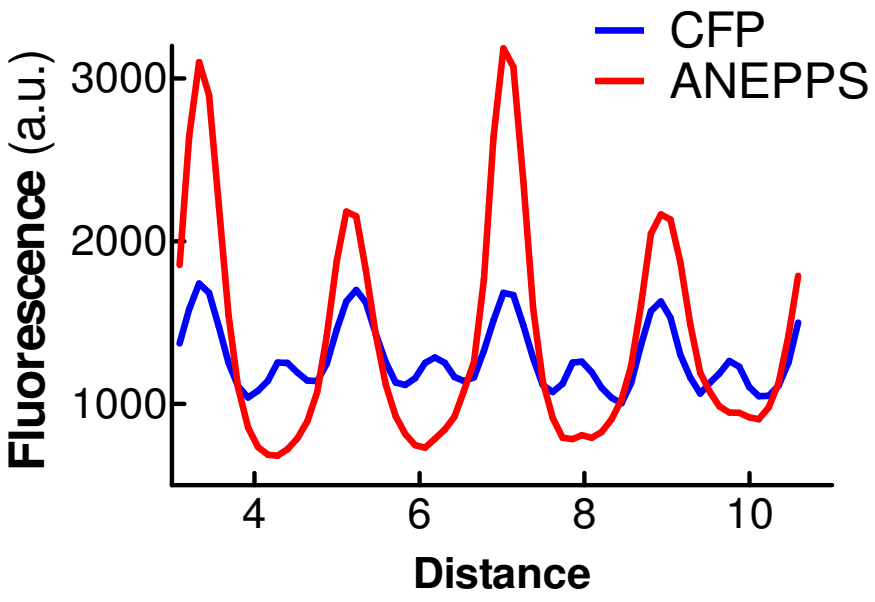
Supplement Material

Supplemental Figure I



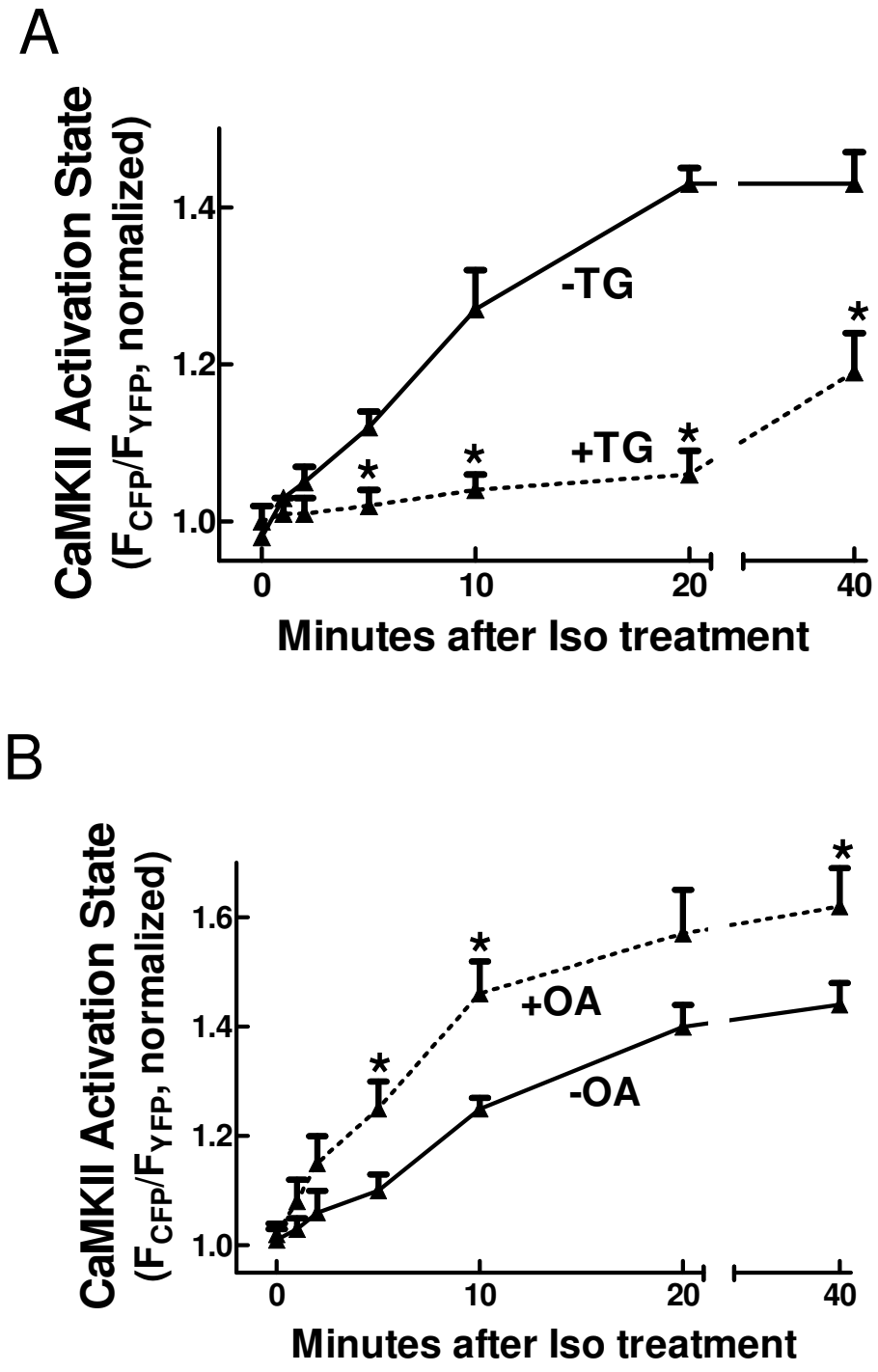
Supplemental Figure I – Change in Camui FRET correlates with autonomous CaMKII activation. (A) Activity assays show significant activation of CaMKIIδ (predominant cardiac form) in all conditions that induced Camui activation. (B) Change in donor to acceptor fluorescence ratio scales linearly with autonomous activity (above background, different treatments indicated by colors) for both the Camui sensor (filled) and CaMKIIδ (open symbols). Direct Ca/CaM activation (black symbols) have higher activity than regression line for given $F/_{CFP}/F_{YFP}$.

Supplemental Figure II



Supplemental Figure II – Camui targeting at the myofilament z-lines was confirmed using di-8-ANEPPS staining of rabbit myocytes. To avoid spectral overlap of Camui and di-8-ANEPPS, high intensity 514nm laser light was used to photobleach YFP. Imaging parameters for Camui and di-8-ANEPPS were respectively 440 and 488 nm for excitation and BP 465-495 and 650-750 for emission. Shown is a sample plot profile of Camui and di-8-ANEPPS fluorescence intensities in the cytosol.

Supplemental Figure III



Supplemental Figure III – Isoproterenol mediated Camui activation in the presence of thapsigargin (TG) and okadaic acid (OA). (A) 10 min pretreatment with 10 μM TG blocks Iso induced Camui activation. (B) 10 min Pretreatment with 1 μM OA partially ablates the slow response of Camui to Iso and increases peak change in FRET. * indicates $p<0.05$ vs. control.