



HHS Public Access

Author manuscript

Nature. Author manuscript; available in PMC 2013 May 08.

Published in final edited form as:

Nature. 2012 November 8; 491(7423): 269–273. doi:10.1038/nature11444.

CaMKII determines mitochondrial stress responses in heart

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Contributions

M.A.J. designed the project, carried out experimental work and wrote the manuscript. O.M.K. and L-S.S. carried out experimental work, analysed data and participated in data interpretation. C.A., Z.G., E.D.L., carried out experimental work and interpreted data. J.L., B.J.H., D.D.H., B.D.F., B.C. and J.Y. carried out experimental work. S.S. provided critical materials and wrote the manuscript. S.A.M. and T.D.S. interpreted data. P.J.M. supervised the research. W.I.S. supervised the research and wrote the manuscript. M.E.A. conceived the project, supervised the research and wrote the manuscript.

Competing financial interests

Dr. Anderson is a named inventor on patents claiming to treat heart disease by CaMKII inhibition. He is a founder and advisor to Allosteros Therapeutics. At present there is no income from these activities.

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Abstract

Myocardial cell death is initiated by excessive mitochondrial Ca^{2+} entry, causing Ca^{2+} overload, mitochondrial permeability transition pore (mPTP) opening and dissipation of the mitochondrial inner membrane potential (Ψ_m)^{1,2}. However, the signaling pathways that control mitochondrial Ca^{2+} entry through the inner membrane mitochondrial Ca^{2+} uniporter (MCU)³⁻⁵ are not known. The multifunctional Ca^{2+} and calmodulin-dependent protein kinase II (CaMKII) is activated in ischemia reperfusion (I/R), myocardial infarction (MI) and neurohumoral injury, common causes of myocardial death and heart failure, suggesting CaMKII could couple disease stress to mitochondrial injury. Here we show that CaMKII promotes mPTP opening and myocardial death by increasing MCU current (I_{MCU}). Mitochondrial-targeted CaMKII inhibitory protein or cyclosporin A (CsA), an mPTP antagonist with clinical efficacy in I/R injury⁶, equivalently prevent mPTP opening, Ψ_m deterioration and diminish mitochondrial disruption and programmed cell death in response to I/R injury. Mice with myocardial and mitochondrial-targeted CaMKII inhibition are resistant to I/R injury, MI and neurohumoral injury, suggesting pathological actions of CaMKII are substantially mediated by increasing I_{MCU} . Our findings identify CaMKII activity as a central mechanism for mitochondrial Ca^{2+} entry and suggest mitochondrial-targeted CaMKII inhibition could prevent or reduce myocardial death and heart failure dysfunction in response to common experimental forms of pathophysiological stress.

Excessive activation of the multifunctional Ca^{2+} and calmodulin-dependent protein kinase II (CaMKII) by Ca^{2+} triggers myocardial death and heart failure^{7,8}, while excessive CaMKII activity promotes multiple defects in myocardial Ca^{2+} homeostasis, including increased mitochondrial Ca^{2+} ^{9,10}. CaMKII inhibition is protective against I/R, MI and neurohumoral toxicity, clinically-relevant forms of myocardial injury marked by disturbed intracellular Ca^{2+} homeostasis^{7,8,11}, but the mechanisms for myocardial protection by CaMKII inhibition are uncertain. Excessive increases in mitochondrial Ca^{2+} lead to mitochondrial permeability transition pore (mPTP) opening and dissipation of the mitochondrial inner membrane potential (Ψ_m)^{1,2}. We first asked if excessive activation of mitochondrial CaMKII could be a mechanism for myocardial dysfunction or death during I/R injury, because I/R injury occurs in the setting of increased mitochondrial Ca^{2+} and because I/R injury is alleviated by Ru360^{12,13}, a selective inhibitor of the mitochondrial uniporter (MCU) current (I_{MCU}) in rats¹², and by CsA, an inhibitor of mPTP, in patients⁶. Here we show that mitochondrial-targeted CaMKII inhibition or treatment with CsA, an mPTP antagonist with clinical efficacy in I/R injury⁶, are both protective against mPTP opening, loss of Ψ_m , mitochondrial disruption and programmed cell death in response to I/R, MI or isoproterenol. The myocardial protective effects of mitochondrial CaMKII inhibition are multivalent and involve increasing mPTP Ca^{2+} tolerance and reduction in I_{MCU} .

In order to test if CaMKII catalytic activity was the mechanism for Ca^{2+} to affect downstream responses to I/R injury, we developed mice with myocardial-delimited CaMKII inhibition by transgenic expression of a membrane-targeted CaMKII inhibitor, CaMKIIN, the most potent and specific CaMKII inhibitory protein¹⁴. We engineered CaMKIIN with a palmitoylation sequence to enhance partitioning into intracellular membranes. We identified CaMKIIN expression in isolated mitochondria of transgenic mice (Supplemental Fig. 1a,b). To determine if CaMKIIN transgenic mice were resistant to I/R injury by a Ca^{2+} -regulated pathway we used isolated, perfused, working mouse hearts to directly measure myocardial

mechanical responses to I/R injury under conditions designed to restrict glycolytic metabolism. WT hearts or hearts with transgenic CaMKIIN expression were perfused with a pyruvate-containing solution (at non-physiological levels) without glucose, so that ATP production relied on oxidative metabolism, and CsA, to prevent mPTP opening, or vehicle (Supplemental Fig. 2a–c). Left ventricular developed pressure (Supplemental Fig. 2b) and the first derivative of left ventricular developed pressure (Supplemental Fig. 3a) were reduced in WT vehicle-treated hearts after I/R injury, but were preserved after I/R injury in hearts with CaMKII inhibition or in WT hearts treated with CsA. Baseline recordings were similar between CaMKIIN-expressing, WT and WT with CsA (Supplemental Fig. 3b). The area of infarcted myocardium following I/R injury (Supplemental Fig. 2d,e) was 66 ± 3.3 percent of the area at risk for WT hearts and was reduced by half with CsA or CaMKIIN expression. The relative area of infarcted myocardium (Supplemental Fig. 2e) was inversely related to the extent of mechanical recovery (Supplemental Fig. 2c), suggesting that the beneficial effects of CsA and CaMKII inhibition ultimately derived from prevention of myocardial death in response to I/R injury. We measured caspase 9, a marker of mitochondrial-triggered apoptosis¹⁵. Caspase 9 was significantly reduced in the CaMKIIN transgenic hearts after I/R injury and in WT hearts treated with CsA (Supplemental Fig. 2f).

We next asked if transgenic expression of CaMKIIN protected mitochondria from I/R injury. Mitochondria are structurally dynamic organelles and loss of the highly ordered internal membrane cristae is an ultrastructural correlate of mPTP opening, loss of Ψ_m and apoptosis initiation¹⁶. We used transmission electron microscopy to examine mitochondrial ultrastructure and to quantify mitochondrial disruption (Supplemental Fig. 2g,h) after I/R injury. Mitochondria of vehicle-treated WT hearts suffered extensive disruption after I/R injury, while CsA significantly protected mitochondria in WT hearts. In contrast, mitochondria from CaMKIIN transgenic hearts were resistant to I/R injury, in the presence or absence of CsA (Supplemental Fig. 2h). These data show that infarct size, mitochondrial structural integrity, mitochondrial-triggered cell death and dysfunction are similarly improved by CsA or CaMKII inhibition, consistent with a concept where CsA and CaMKII both engage a mitochondrial pathway leading to mPTP opening during pathological stress.

In order to better understand the protective effects of mitochondrial CaMKIIN expression, we measured Ca^{2+} -induced injury in isolated mitochondria. Loss of mitochondria function by mPTP opening can be measured in isolated mitochondria in response to supraphysiological increases in mitochondrial Ca^{2+} ¹⁷. Decreases in light scattering is a correlate of mPTP opening¹⁸. We found that mitochondria with CaMKIIN expression were resistant to Ca^{2+} -triggered increases in light scattering compared to WT controls (Fig. 1a). We corroborated our findings with Ca^{2+} -induced mitochondria light scattering by a similar assay where a decrease in fluorescence corresponds to a loss of Ψ_m . We loaded mitochondria isolated from WT and CaMKIIN transgenic mice with a voltage-sensitive fluorescent indicator, JC-1¹⁶, to test whether CaMKIIN expression affected Ψ_m responses to Ca^{2+} . Mitochondria isolated from WT hearts were significantly more sensitive to Ψ_m loss in response to a lethal Ca^{2+} challenge compared to mitochondria with CaMKIIN expression (Fig. 1b). CsA treatment preserved the Ψ_m during exogenous Ca^{2+} challenge in mitochondria isolated from WT, while mitochondria derived from CaMKIIN transgenic hearts were protected even in the absence of CsA (Fig. 1c).

We detected CaMKII associated with mitochondria and mitoplasts, inner mitochondrial membrane vesicles from osmotically-shocked isolated cardiac mitochondria (Supplemental Fig. 4c,d), similar to previous studies^{19,20}. However, CaMKII may contribute to Ca²⁺ overload and myocardial death by actions at extra-mitochondrial Ca²⁺ homeostatic proteins²¹. Therefore we developed transgenic mice with CaMKIIN expression limited to myocardial mitochondria (mtCaMKIIN) to test if CaMKII inhibition targeted to mitochondria affected mitochondria susceptibility to excess Ca²⁺. Unlike CaMKIIN transgenic mice, hearts expressing mtCaMKIIN have expression restricted to mitoplasts (Supplemental Fig. 1b). Mitochondria isolated from mtCaMKIIN hearts showed no difference in baseline metabolic function compared to WT littermates (Supplemental Fig. 5). We measured mitochondrial Ca²⁺ uptake with a membrane-impermeable indicator, Ca²⁺ Green-5N⁴ and found cardiomyocytes from mtCaMKIIN transgenic mouse hearts had significantly slower mitochondrial Ca²⁺ uptake, measured as a rate of decrease in Ca²⁺ Green-5N fluorescence, than mitochondria of WT cardiomyocytes (Fig. 1d,e). We interrogated the overall Ca²⁺ capacity of myocardial mitochondria with serial additions of Ca²⁺, and found that mtCaMKIIN expression increased the mitochondrial Ca²⁺ capacity prior to mPTP opening (Supplemental Fig. 6a,b). We next measured mitochondrial Ca²⁺ concentration changes in HeLa cells in response to ATP-evoked Ca²⁺ release using a GFP-based Ca²⁺ indicator, cameleon²², designed to monitor Ca²⁺ concentration within the mitochondria. Our results show reduced ATP-triggered Ca²⁺ entry into mitochondria in HeLa cells co-transfected with mitochondrial-directed cameleon and mtCaMKIIN compared with control cells without mtCaMKIIN expression (Fig. 1f & Supplemental Fig. 7a,b). These data suggest that the benefits of CaMKII inhibition to protect against mitochondrial stress derive from reducing mitochondria Ca²⁺ entry and enhancing mPTP Ca²⁺ tolerance.

The MCU is a major Ca²⁺ entry pathway through the mitochondrial inner membrane³⁻⁵ and isolated cardiomyocytes²³ and perfused hearts^{12,13} are resistant to opening and cell death after Ca²⁺ challenge in the presence of I_{MCU} inhibitor drugs. To examine potential effects of CaMKII on the MCU, we performed voltage (patch) clamp studies on mitoplasts. We measured an Ru360-sensitive mitochondrial Ca²⁺ current, that was activated by Ca²⁺ (K_{1/2} = 23 mM), consistent with known properties of I_{MCU}³ (Fig. 2a & inset). Furthermore, CsA had no effect on I_{MCU} (Supplemental Fig. 8a), indicating that the beneficial actions of CaMKII inhibition on mPTP were distinct from the effects of CaMKII on the MCU. I_{MCU} was increased by addition of a constitutively active, monomeric CaMKII mutant (T287 to D, T/D) to the pipette solution (equivalent to the mitochondrial matrix) in the presence of ATP (Fig. 2b & Supplemental Fig. 8b). Addition of T/D CaMKII did not increase I_{MCU} in the presence of a non-hydrolyzable ATP analog, adenosine 5'-(β, γ-imido) triphosphate tetralithium salt (Fig. 2b), or in the absence of ATP (not shown). Furthermore, a catalytically incompetent CaMKII mutant (K43 to M, K/M) failed to increase I_{MCU} in the presence of activating ATP and calmodulin (Fig. 2b & Supplemental Fig. 8b). These findings indicate that the catalytic activity of CaMKII was necessary to increase I_{MCU}. Inhibiting serine/threonine phosphatases with calyculin A increased I_{MCU} (Fig. 2c & Supplemental Fig. 8c), suggesting that endogenous and exogenous CaMKII were capable of increasing I_{MCU} under appropriate experimental conditions. We next measured I_{MCU} from CaMKIIN and mtCaMKIIN heart mitochondria to test if CaMKIIN expression in mitoplasts affected Ca²⁺

uptake. I_{MCU} was lower in CaMKIIN-expressing mitoplasts using Ca^{2+} as the charge carrier (Fig. 2d & Supplemental 8d). In contrast, when Na^{+} was substituted for Ca^{2+} , a condition that does not support CaMKII activation, the differences in WT and CaMKIIN-expressing mitoplasts were no longer apparent, indicating that CaMKIIN was unlikely to affect MCU expression. Ru360 nearly and equivalently eliminated I_{MCU} in all genotypes, suggesting that MCU antagonist sensitivity was not affected by CaMKIIN (Fig. 2d & Supplemental Fig. 8a). CaMKII and MCU co-immunoprecipitated from myocardial mitochondria lysate (Fig. 2e), suggesting that CaMKII is associated with MCU in heart. CaMKII enhances Ca^{2+} currents through sarcolemmal voltage-gated Ca^{2+} channels ($Ca_v1.2$)²⁴ and intracellular Ca^{2+} -gated ryanodine receptor Ca^{2+} release channels²⁵ by phosphorylation of defined serines/threonines. We next identified two putative CaMKII target sites on MCU, serines 57 and 92, and mutated them to alanines (SS/AA), to test for a direct effect of CaMKII on MCU. We over-expressed WT and SS/AA MCU in HEK cells and measured I_{MCU} from isolated mitoplasts. Constitutively active, monomeric CaMKII (T/D) added to the pipette solution failed to increase I_{MCU} in mitochondria from cells transfected with SS/AA MCU compared to WT MCU transfections (Fig. 2f & Supplemental Fig. 8e). In contrast, CaMKII added to the bath solution failed to affect I_{MCU} recorded from WT or SS/AA over-expressing mitoplasts (not shown). These studies, using a CaMKII-resistant SS/AA MCU mutant, suggest that the N-terminus of MCU is oriented in the mitochondrial matrix⁴. Taken together, these data show that CaMKII interacts with MCU and promotes mitochondrial Ca^{2+} entry, likely by catalyzing phosphorylation of serines 57 and 92, consistent with the concept that MCU is an important CaMKII target in mitochondria.

We next used our mtCaMKIIN mice to determine if mitochondrial CaMKII inhibition was sufficient to explain the protective effects of CaMKII inhibition on I/R injury in isolated perfused hearts in the absence of glucose (Supplemental Fig. 2)^{7,11}. We found that hearts from mtCaMKIIN mice had preserved mechanical function (Fig 3a), reduced MI size (Fig 3b,c), preserved mitochondrial integrity (Fig. 3d,e) and reduced caspase 9 activity (Fig. 3f) compared to WT littermate control hearts after I/R injury. Baseline left ventricular developed pressures were similar between WT and mtCaMKIIN hearts (Supplemental Fig. 9). We found no difference in left ventricular mechanical recovery ($p = 0.976$) between WT and mtCaMKIIN hearts treated with CsA, and CsA added no further protection for mtCaMKIIN hearts subjected to I/R injury ($p = 0.946$). Neurohumoral toxicity is a major cause of adverse outcomes after MI, including heart failure²⁶, and β adrenergic receptor antagonist drugs are a therapeutic mainstay for reducing myocardial death and dysfunction after ischemic injury and MI²⁶. CaMKII is activated by isoproterenol in heart and CaMKII inhibition protects against isoproterenol-mediated myocardial injury^{7,8}. The protective effects of mtCaMKIIN expression were also evident *in vivo*, because mtCaMKIIN mice showed fewer TUNEL-stained nuclei than WT controls 5 h after a MI (Fig 4a,b) or 30 min after isoproterenol treatment (Fig. 4b). Thus, mitochondrial targets appear to be essential for the cardioprotective benefits of CaMKII inhibition in a diverse set of clinically-relevant injury models.

Our findings identify CaMKII as a modulator of mitochondrial Ca^{2+} homeostasis and a crucial component of a final pathway in heart disease due to ischemia and neurohumoral

toxicity, by showing that CaMKII increases I_{MCU} and that CaMKII inhibition reduces I_{MCU} and the mPTP opening. Our study suggests that CaMKII inhibition could reduce adverse responses, including death, to common forms of pathological myocardial stress by multivalent actions on mitochondria, including I_{MCU} inhibition and blunting Ca^{2+} responsiveness of mPTP.

Methods

Transgenic mice

Animals were maintained in the University of Iowa Animal Facility and treated in accordance with Institutional Animal Care and Use Committee guidelines (PHS Animal Welfare Assurance, A3021-01). CaMKIIN transgenic mice (C57BL/6 background) were generated as described¹⁴. Targeting of β CaMKIIN (accession NM_033259.2) to mitochondria in transgenic mice was achieved by fusion of the *cox8a* N-terminus. The N-terminal 28 amino acids of *cox8a* were added to the 5' of HA- β CaMKIIN sequence by PCR using the 5' primer: CGGTCGACATGTCCGTCCTGAC and the 3' primer ATGTCGACTTCTCCCGGCGGCA (Integrated DNA Technologies) with *Sall* sites at the extreme ends. The product (~100 base pairs) was purified from agarose gel (Quiagen gel extraction kit) and was digested with the restriction enzyme, *Sall*. The resulting DNA product was ligated into the *Sall* site 5' of the HA sequence of pc-MHC-script-Hgh-HA-tagged CaMKIIN vector. Mouse embryonic stem cells were injected with the linearized DNA (digested with *NotI*) in the University of Iowa Transgenic Mouse Core Facility and implanted into B6D2 pseudo-pregnant females. Insertion of the transgene into the mouse genome was confirmed by PCR analysis (not shown) using the forward primer, cgataaacttatgtccgagatccta and reverse primer, gtcactctgatcaccactctct, producing a product of 196 bases. Mice were backcrossed to F4 generation or greater into the C57 background. Transgenic and control mice of either gender were sacrificed at the age of 2-3 months.

Isolated perfused working mouse hearts

Langendorff-perfused mouse hearts were used to measure left ventricular function and myocardial viability, as described²⁷. Mice were anesthetized with avertin and hearts were quickly excised and rinsed in iced Tyrode's solution containing (in mM): 137 NaCl, 5.4 KCl, 0.5 MgCl₂, 0.16 NaH₂PO₄, 3 NaHCO₃, 5 HEPES-NaOH, and Heparin 1 mg/10 ml, which was previously adjusted to a pH of 7.4 with NaOH at room temperature. All chemicals were from sigmaaldrich.com, unless otherwise noted. Excess tissue was dissected away. Hearts were perfused retrogradely through the aorta for 1–2 minutes at room temperature with Hanks' balanced salt solution (GIBCO, Cat No. 14025-092) and mounted on a modified Langendorff apparatus (HSE-HA perfusion systems, Harvard Apparatus) for retrograde aortic perfusion at a constant pressure of 80 mm Hg with carbogen (95% O₂, 5% CO₂) and Krebs–Henseleit bicarbonate (KHB) solution consisting of (in mM): 25 NaHCO₃, 118 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.2 NaH₂PO₄, 2.5 CaCl₂, 0.5 Na-EDTA, 2 pyruvate, with pH equilibrated to 7.4 with 5 μ M CsA or DMSO for control. Hearts were perfused for 10 min to ensure metabolic equilibrium followed by 20 or 30 min of global ischemia then 50 min of reperfusion. Measurements were recorded from each heart for the entire time of perfusion.

Heart infarct size and caspase 9 activity assay

Following the I/R protocol shown in Supplemental Figure 2, hearts were immediately frozen. Three-0.5 mm sections were cut from frozen hearts, labeled with 5% (wt/vol) 2,3,5-triphenyltetrazolium chloride (TTC) in PBS buffer and fixed in 10% formalin. Caspase 9 activity assays were performed on tissue lysate following manufacturers protocol (Invitrogen.com) and normalized to protein concentration ($\mu\text{g/ml}$).

Transmission electron microscopy

Hearts were fixed in 2.5 % glutaraldehyde and 0.1 M Na cacodylate buffer overnight at 4°C, washed 3 X 20 min in 0.1 M Na cacodylate buffer, pH 7.2, fixed in 4 % OsO₄, washed in 0.1 M Na cacodylate buffer then dH₂O, 2.5 % uranyl acetate, EtOH series to dehydrate then EtOH and Spurr's with final solution 100% Spurr's. Hearts were embedded in Spurr's at 60°C for 24 – 48 h. Ultramicrotomy at 90 nm and samples collected on 200 mesh for formvar grids for staining with uranyl and lead. Stained sections were examined with a JEOL JEM-1230 and digital images were collected with a Gatan UltraScan 1000 2k×2k CCD camera.

Mitochondrial isolation and subcellular fractionation

Isolation of mitochondria was performed on ice. Freshly harvested mouse hearts were injected with 1.0 % protease XXIV in MSE buffer (in mM, 5 MOPS, 70 sucrose, 2 EGTA, 220 mannitol, pH 7.2 with KOH) then homogenized. HEK cells were washed then homogenized. Nuclei and unbroken cells were pelleted by centrifugation at 600 Xg for 10 min twice. The crude mitochondrial and cytosolic fraction was obtained from the supernatant by centrifugation at 8,500 Xg for 20 min. The pellet was further purified with a 40% percoll gradient, washed twice in MSE then resuspended in 200 μl MSE with protease inhibitors. For mitoplast preparation, mitochondria were resuspended in hypotonic solution (in mM, 5 sucrose, 5 HEPES and 1 EGTA (pH 7.2 with KOH) for 5 min to remove outer membrane. After sedimenting for 5 min at 4,000Xg mitoplasts were resuspended in (in mM) 750 KCl, 100 HEPES and 1 EGTA (pH 7.2 with KOH).

MCU current recording (whole-mitoplast configuration)

Signals were measured with an Axon 200B patch-clamp amplifier controlled by a personal computer using a Digidata 1320A acquisition board driven by pClamp 8.0 software (Axon Instruments). For formation of gigaohm seals and initial break-in to the whole-cell voltage clamp configuration, mitoplasts were perfused with solution containing (mM): 150 Na gluconate, 10 Hepes and 0.2 CaCl₂ (pH 7.4, adjusted with NaOH). Bath solutions with different Ca²⁺ concentrations were made by adding the proper of volume of 1 M CaCl₂. Pipettes, after filling with solution (mM) Na gluconate, 5 NaCl, 135 sucrose, 10 HEPES and 1.5 EGTA (pH7.2 with NaOH) had an access resistance of 25–35 MOhm³. After rupturing of mitoplast membranes the capacitance was from 5 to 9 pF. A ramp voltage command protocol from -160 to 80 mV, for 2 seconds, was applied from a holding potential of 0 mV to evoke currents. Reagents and enzyme added (as noted in text) were 100 μM ATP, 100 μM non-hydrolysable ATP, 2 μM CaM, 0.5 μM CaMKII (WT and mutants; T/D and K/M), 10 nM Ru360 and 5 μM CsA.

Mitochondrial Ψ m measurement

Isolated mitochondria were loaded with JC-1 following manufacturers instructions (Invitrogen.com) and placed in a 96 well black-walled plate with 0.2 μ g/ml JC-1 in buffer with DMSO, 0.2 μ M CsA or 2.5 μ M carbonyl cyanide *p*-[trifluoromethoxy]-phenyl-hydrazine (FCCP). At the indicated time point 200 μ M CaCl_2 was added to the suspension. Fluorescent measurements were taken according to the manufacturer's protocol. Light scattering was assessed after adding CaCl_2 (free Ca^{2+} was calculated using the Maxchelator at <http://www.stanford.edu/~cpatton>) by decreased absorbance over 30 min at 540 nm using a F200 96-well plate reader (Tecan) in swelling buffer (in mM, 200 sucrose, 0.002 EGTA, 5 succinate, 0.0002 rotenone, 20 Tris, 20 Hepes, 1 KH_2PO_4 and 1 μ g/ml oligomycin, pH 7.2), with a use gain of 100. Data were normalized to the initial measurement and plotted using Prism Graph.

Ca^{2+} retention assay

CaG5N was used to monitor extramitochondrial Ca^{2+} in saponin-permeabilized, isolated adult mouse cardiomyocytes. The ventricular cardiomyocytes were isolated from adult mice as described²⁸. Recordings of cardiomyocyte mitochondrial Ca^{2+} uptake were performed in a 96 well plate with 100 μ l of respiration buffer (in mM, 100 KAsp, 20 KCl, 10 HEPES, 5 glutamate, 5 malate, and 5 succinate pH 7.3) supplemented with 100 μ M blebbistatin, 5 μ M thapsigargin, 0.005% saponin and 1 μ M Ca^{2+} green-5N (CaG5N, Invitrogen.com) with freshly isolated cardiomyocytes. CaG5N fluorescence was monitored at excitation, 485 nm; emission, 535 nm after adding CaCl_2 (100 μ M free Ca^{2+} at 3 min intervals at 30°C). Cyclosporin A (5 μ M) and Ru360 (0.1 μ M) were used to block the mitochondrial permeability transition pore and Ca^{2+} uniporter, respectively.

MI and Isoproterenol-treated mice

Assays are described in previous publications⁸.

Transmission immuno-electron microscopy

Isolated mitochondria fixed in EM grade 4% paraformaldehyde with 0.05% glutaraldehyde in PBS pH 7.4 for 1 h at 4°C were processed and labeled with antibodies at 1:250 (CaMKII, CoxIV, rabbit IgG and HA).

Immunoprecipitation, Western and activity assays

Immunoprecipitation was performed over 1 h with mitochondrial lysate (200 μ g protein) and protein A magnetic beads. SDS-PAGE and Western blotting studies used lysate of heart subcellular fractions. Protein content was determined with a Bradford assay. Samples (25 μ g) were separated on NuPAGE 4–12% Bis-Tris gels (Invitrogen.com) and transferred to PVDF membranes. After blocking with Odyssey blocking buffer (LI-COR Biosciences), the membranes were incubated with the following primary antibodies (dilutions): CaMKII (1:500), SERCA2 (1:250), calnexin (1:250), CoxIV (1:500) ATP5a (1:500), MCU (1:500), nucleolin (1:100), mfn1 (1:500) and HA (1:1000). Appropriate secondary antibodies were used and bands visualized. Activity assays²⁹ were done using mitochondria lysate with biotin capture membrane (Promega).

Mitochondrial respiration

State 3 and state 4 respiration were measured by polarography in a 600- μ l incubation chamber with stir bar and oxygen electrode as we have accomplished in past studies³⁰. The ADP/O ratio was calculated as molar ADP added divided by molar oxygen consumed during conversion to ATP as we described previously³⁰. Respiratory coupling was assessed by simultaneous determination of respiration and membrane potential as described³⁰.

Statistical analysis

Statistical analysis was performed using the analysis of variance (ANOVA or Student's t-test, as appropriate). Post hoc pairwise comparisons were performed using Bonferroni's multiple-comparison test (Prism graph), as appropriate. Other tests were done as noted in the text using Prism graph. Results were given as means \pm s.e.m. A p value < 0.05 was considered significant. n.s. = not significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

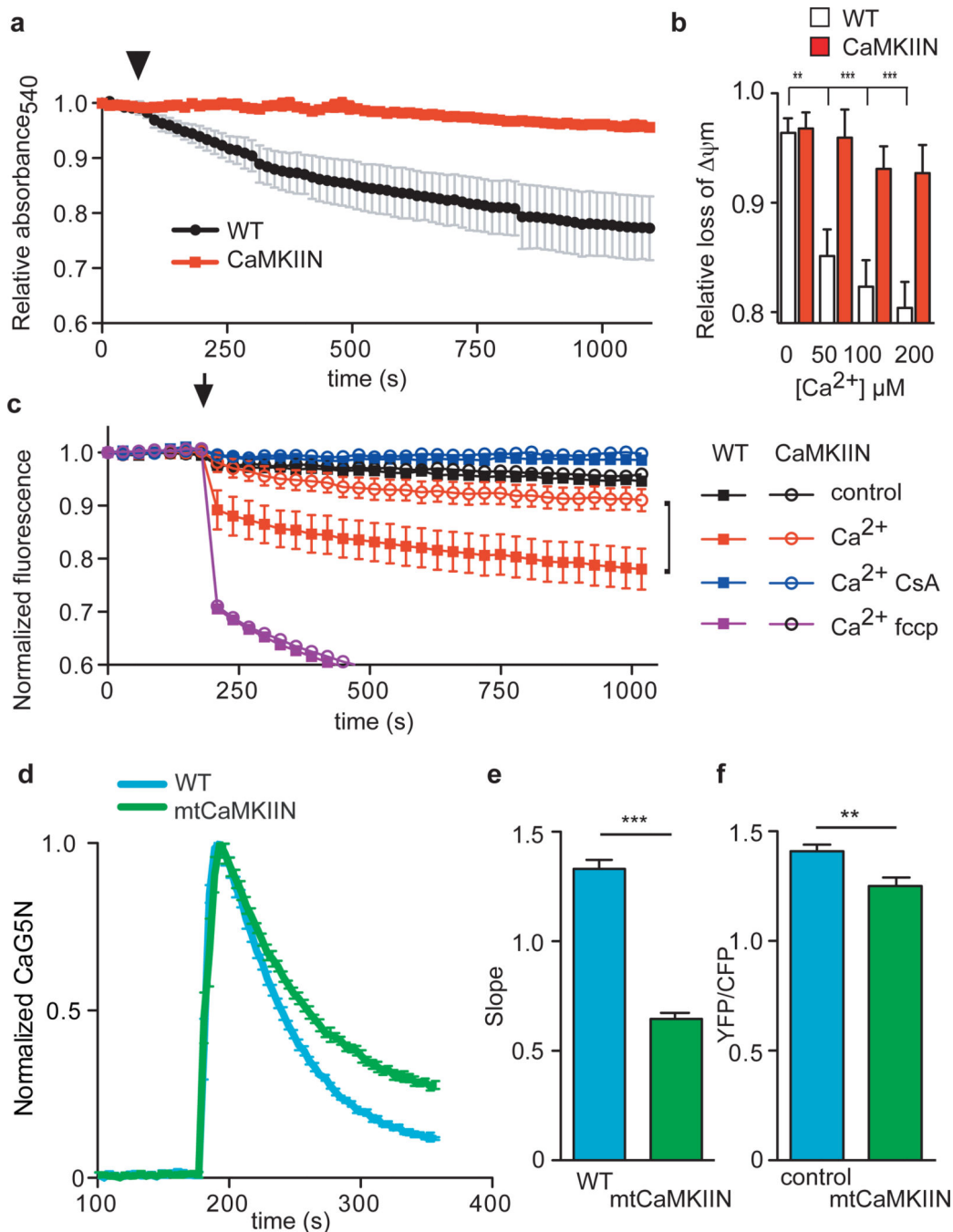
Acknowledgments

The authors wish to acknowledge discussions with Dr. Amy Lee, Dr. Beverly Davidson, Dr. Kevin Campbell and Dr. Curt Sigmund (University of Iowa). This work was funded by AHA 0635357N (M.A.J.); NIH R01 HL090905 (L.-S.S); NIH R01 HL079031, R01 HL62494, R01 HL70250 and R01 HL113001 and supported by a grant from the Fondation Leducq for the Alliance for CaMKII Signaling (M.E.A.); NIH R01 HL084583 and R01 HL083422 and Pew Scholars Trust (P.J.M.).

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**Figure 1.**

Isolated mitochondria with transgenic, membrane-targeted CaMKII inhibition (CaMKIIN) are resistant to Ca²⁺ (200 μ M Ca²⁺ free) challenge. **a.** Mitochondria mPTP Ca²⁺-dependent (arrow) opening reflected by a decrease in light scattering corresponding to an increase in mitochondrial volume, $n = 3$ /genotype. **b.** Increasing [Ca²⁺] promotes loss of $\Delta\psi_m$ more in mitochondria isolated from WT than CaMKIIN mouse hearts (** $p < 0.001$, *** $p < 0.0001$, $n = 3$ hearts/group with duplicate measurements). **c.** Inner mitochondrial membrane potential ($\Delta\psi_m$) measurements in isolated mitochondria using a fluorescent reporter, JC-1. Reduced

signal from baseline after addition of Ca^{2+} (200 μM free Ca^{2+} , at arrow) indicates loss of Ψm ($p < 0.03$ for all time points after the Ca^{2+} challenge) between WT and CaMKIIN treated with Ca^{2+} alone (indicated with black bracket), red squares versus open red circles, $n = 5$ hearts/group with duplicate measurements. **d.** Normalized traces showing rate of mitochondrial Ca^{2+} uptake in saponin-permeabilized cardiomyocytes after the addition of Ca^{2+} (arrow, 100 μM free Ca^{2+}). Mitochondrial Ca^{2+} uptake was monitored in cells by loss of Ca^{2+} Green-5N fluorescence. The rate of decline in fluorescence reflects the rate of mitochondrial Ca^{2+} uptake. **e.** Summary data show the rate of mitochondrial Ca^{2+} uptake. Nonlinear regression fits for mitochondria Ca^{2+} uptake between WT and CaMKIIN cardiomyocytes ($***p < 0.0001$, $n = 6/\text{genotype}$). **f.** Summary data show reduced Ca^{2+} uptake in mitochondria, measured using a cameleon mitochondrial-targeted Ca^{2+} indicator, from HeLa cells expressing mtCaMKIIN compared to myc-expressing controls ($**p = 0.003$, $n = 23$ cells/group). Data represent mean \pm s.e.m.

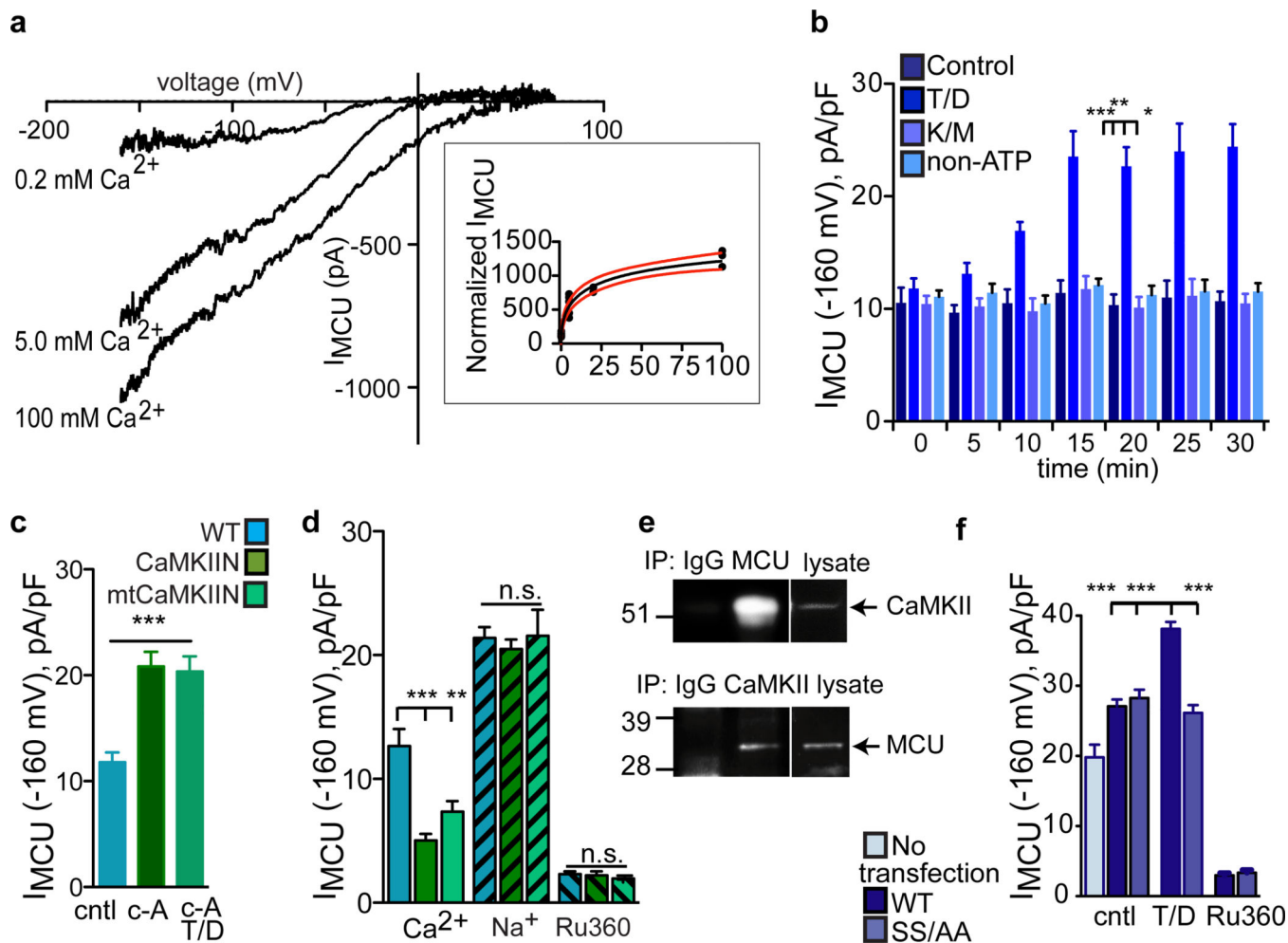


Figure 2.

CaMKII agonist actions on I_{MCU} require serines 57 and 92. **a.** I_{MCU} is a Ca^{2+} -dependent conductance. Inset shows I_{MCU} in 0.2, 5 and 100 mM bath [Ca^{2+}] fit with the Hill equation (standard slope). $V_{1/2} = 23.8$ mM, $R^2 = 0.955$ and $h = 0.057$. Red lines show the 95% confidence intervals (runs test $p = 0.743$). **b.** Summary data and time course for I_{MCU} recorded with 0.2 mM Ca^{2+} after obtaining a high resistance seal and mitoplast membrane rupture (time 0) allowing dialysis of CaMKII T/D. Replacing ATP with non-hydrolyzable ATP (non-ATP) analog (both at 0.1 mM) does not allow a CaMKII-dependent increase in I_{MCU} (all mutant CaMKII at 0.5 μM); T/D and ATP; T/D non-ATP; kinase inactive CaMKII (K/M), CaM and ATP, $n = 6$ (WT), 7 (T/D), 6 (K/M) and 5 (non-ATP), * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ at 20 min. **c.** Summary data for I_{MCU} recorded with 0.2 mM Ca^{2+} after addition of 100 nM calyculin A (c-A); c-A with T/D CaMKII (0.5 μM), ATP. $n = 7$ (control), 9 (C-a) and 7 (C-a with T/D), * $p < 0.0001$. **d.** Summary data for I_{MCU} from WT, CaMKIIN and mtCaMKIIN mitochondria. Na^+ current (150 mM) recorded in the absence of bath (intermembrane space equivalent) Ca^{2+} or Ru360 (10 nM). ** $p < 0.001$, *** $p < 0.0001$, $n = 7$ (cntl groups), 8 (T/D, WT), 9 (T/D, SS/AA) and 6 (Ru360 groups). **e.** MCU and CaMKII co-immunoprecipitate from mitochondrial lysate. **f.** Summary data for I_{MCU} recorded from HEK cell mitoplasts with and without transfection of WT or SS/AA MCU

mutants. HEK mitoplast Ca^+ currents were inhibited by Ru360 (10 nM). For Ca^{2+} $n = 10$ (WT and CaMKIIN) and 7 (mtCaMKIIN); Na^+ $n = 15$ (WT), 13 (CaMKIIN) and 12 (mtCaMKIIN) and for Ru360 $n = 8$ (WT and CaMKIIN), 5 (mtCaMKIIN), *** $p < 0.0001$. Data represent mean \pm s.e.m, except for inset (see **a**).

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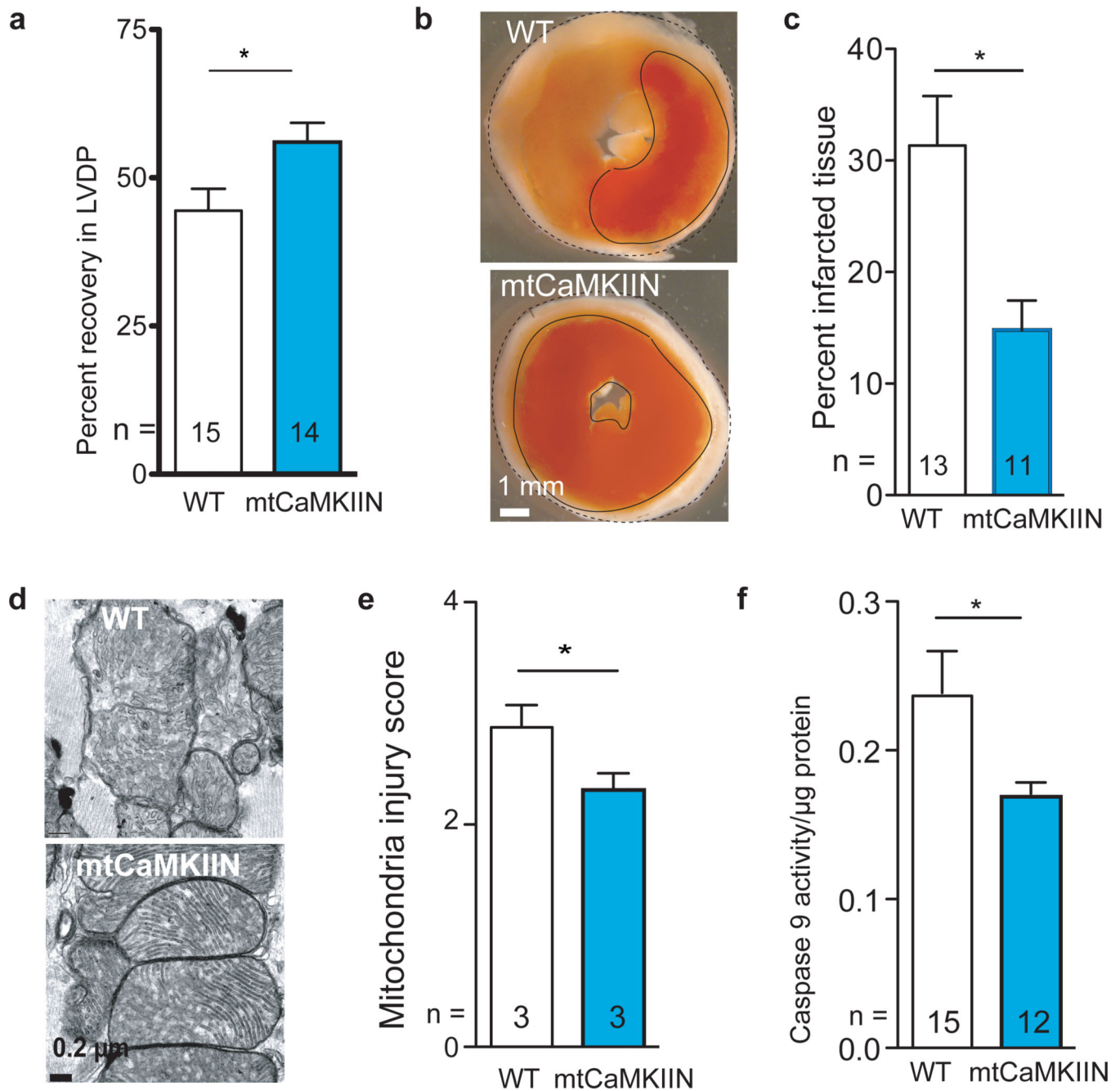


Figure 3. mtCaMKIIN hearts are resistant to I/R injury. **a.** LVDP recovery following I/R as a percentage of the baseline value (* $p = 0.026$). **b.** Representative TTC stained heart sections. The dark red staining represents living myocardium and the solid black outlines form boundaries demarcating viable from dead tissue. **c.** Summary data from TTC stained hearts showing relative area of infarct normalized to WT, measured as in Supplemental Fig. 2e, * $p = 0.006$. **d.** Representative TEM images from hearts treated as in panel **a.** **e.** Summary mitochondria injury scores for TEM studies by the criteria used in Supplemental Fig. 2h. (* $p = 0.003$, more than 500 mitochondria from at least 10 random fields were counted/

genotype). **f.** Caspase 9 activity from hearts treated as in panel **a** (* $p = 0.033$, $n =$ number of hearts/genotype). Data represent mean \pm s.e.m.

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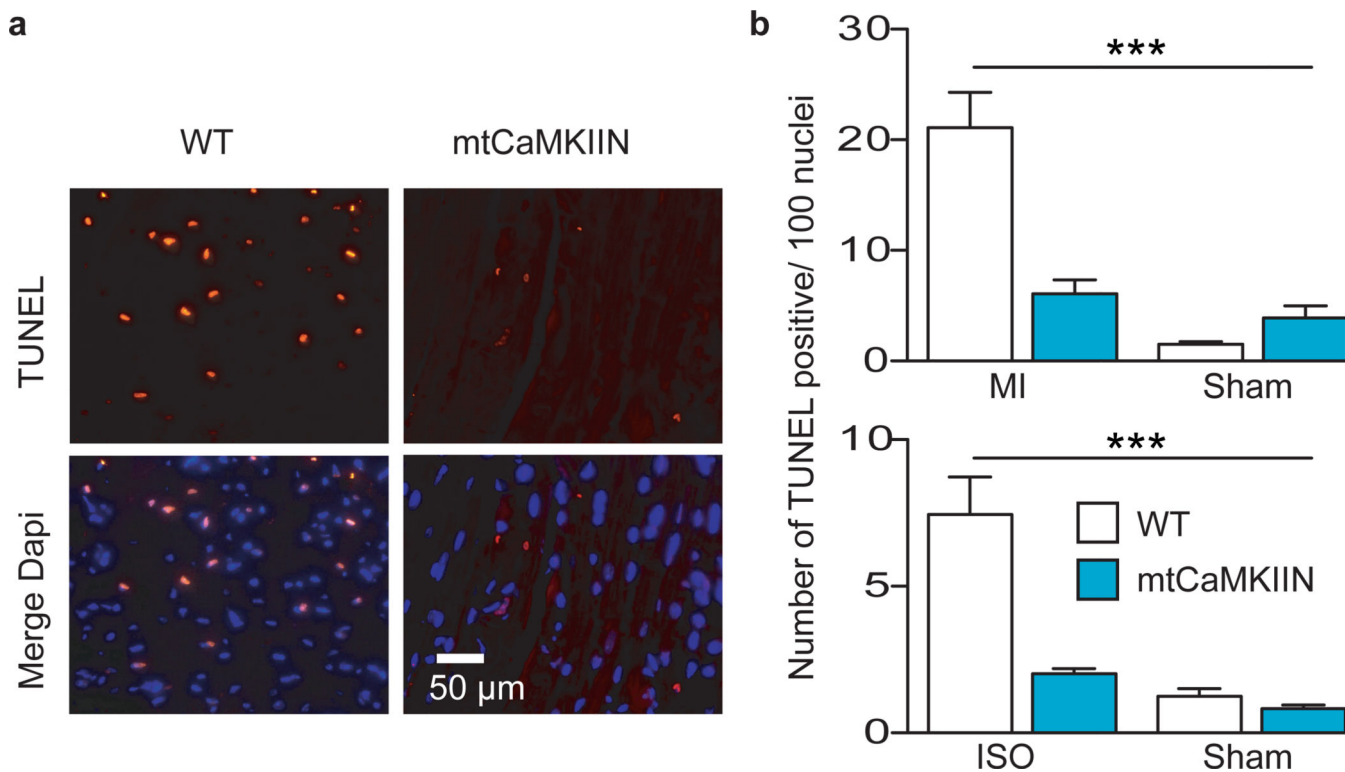


Figure 4.

mtCaMKIIN hearts are resistant to apoptosis *in vivo*. **a**. Representative images of TUNEL-stained nuclei from WT and mtCaMKIIN heart transverse sections 5 h after MI. Dapi stain shows total number of nuclei. Scale bar indicates 50 μ m. **b**. Upper panel - Summary data for the number of TUNEL-stained nuclei from WT and mtCaMKIIN heart sections 5 h after MI (3 hearts/genotype, 10 images/heart). Lower panel - Summary data for the number of TUNEL-stained nuclei from WT and mtCaMKIIN heart sections 30 min after isoproterenol (ISO) treatment (15 mg/kg, 3 hearts/genotype, 10 images/heart, *** $p < 0.0001$). Data represent mean \pm s.e.m.