# Mitochondrial $Ca^{2+}$ uniporter and CaMKII in heart

**ARISING FROM** M. A. Joiner *et al. Nature* **491,** 269–273 (2012); doi:10.1038/nature10234

The influx of cytosolic $Ca^{2+}$ into mitochondria is mediated primarily by the mitochondrial calcium uniporter (MCU)[1], a small-conductance, $Ca^{2+}$-selective channel[2–6]—MCU modulates intracellular $Ca^{2+}$ transients and regulates ATP production and cell death[1]. Recently, Joiner *et al.* reported that MCU is regulated by mitochondrial CaMKII, and this regulation determines stress response in heart[7]. They reported a very large current putatively mediated by MCU that was about two orders of magnitude greater than the MCU current ($I_{MCU}$) that we previously measured in heart mitochondria[3]; furthermore, the current traces presented by Joiner *et al.* showed unusually high fluctuations incompatible with the low single-channel conductance of MCU. Here we performed patch-clamp recordings from mouse heart mitochondria under the exact

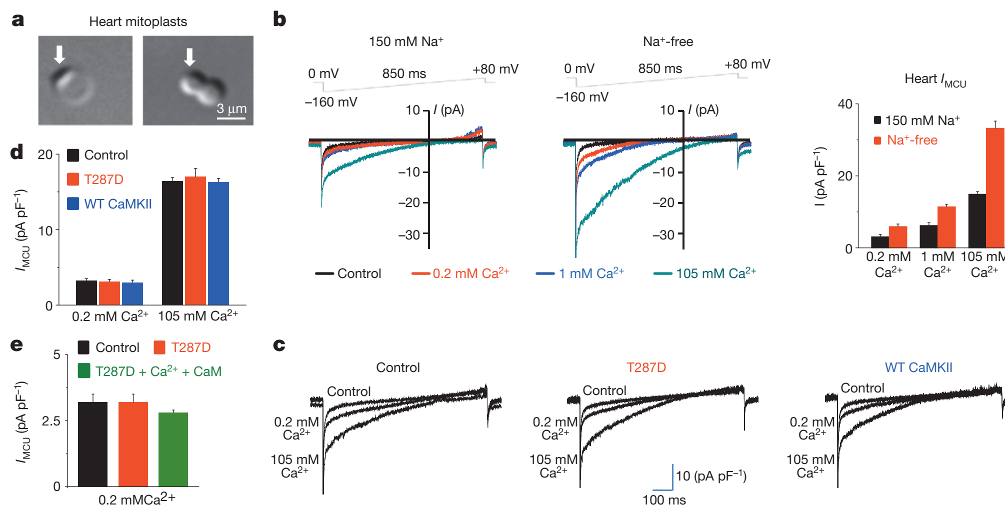


**Figure 1 | Heart MCU current and CaMKII. a**, Transmitted image of heart mitoplasts obtained by exposure of mitochondria to 5-min hypotonic shock. Both round (left) and 'figure-8-shaped' (right) mitoplasts were present in this preparation and used for electrophysiological experiments. Arrows indicate remnants of the outer mitochondrial membrane. The average diameter of heart mitoplasts in this preparation is ~4.5 µm ($n = 65$), which corresponds well with the average membrane capacitance ($C_m$) measurements of 0.67 pF that we previously reported. **b**, Representative heart whole-mitoplast MCU currents ($I_{MCU}$) recorded in the presence (left) or absence (middle) of 150 mM Na-gluconate in both the pipette and bath solutions. $I_{MCU}$ was recorded with different bath $Ca^{2+}$ concentrations: 0.2 mM (red), 1 mM (blue) and 105 mM (green). $I_{MCU}$ was blocked by 50 nM RuR added to the 0.2 mM $Ca^{2+}$ bath solution (control, black). Currents in left and middle panels are not normalized and were recorded from two different mitoplasts with comparable membrane capacitance ($C_m$ = 0.80 pF and 0.84 pF, respectively). The voltage ramp protocol used to elicit $I_{MCU}$ is indicated at the top. With $Na^+$ in the recording solutions we also observed a small outward current at high positive voltages. This current was absent in $Na^+$-free conditions (middle and ref. 3). Pipette solution (in mM): 150 Na-gluconate, 40 HEPES, 2 NaCl, 1.5 EGTA, tonicity 450 mmol per kg with sucrose, pH 7.2 with NaOH. Bath $Ca^{2+}$ solutions with 0.2 and 1 mM $Ca^{2+}$ were prepared by addition of 1 M stock solution of $CaCl_2$ into the bath solution containing (in mM): 150 Na-gluconate, 40 HEPES, tonicity 300 mmol per kg, pH 7.4 with NaOH. The bath solution with 105 mM $Ca^{2+}$ contained 105 mM $CaCl_2$ and 10 mM HEPES, pH 7.2 with Tris base. Right: histogram representing average MCU current densities ($I_{MCU}$ normalized to $C_m$) obtained in the presence (black) or absence (red) of 150 mM Na-gluconate in recording solutions with different bath $Ca^{2+}$ concentrations (0.2, 1 and 105 mM). Current amplitudes were measured at 5 ms after stepping from 0 to –160 mV. $I_{MCU}$ densities were as follows: at bath 0.2 mM $Ca^{2+}$, 3.3 ± 0.4 pA $pF^{-1}$ ($n = 8$) with 150 Na-gluconate in recording solutions and 6 ± 0.7 pA $pF^{-1}$ without Na-gluconate in recording solutions; at bath 1 mM $Ca^{2+}$, 6.2 ± 0.7 pA $pF^{-1}$ ($n = 9$) with Na-gluconate and 11.4 ± 0.7 pA $pF^{-1}$ ($n = 6$) without Na-gluconate; at bath 105 mM $Ca^{2+}$, 14.2 ± 0.7 pA $pF^{-1}$ ($n = 12$) with Na-gluconate and 33.2 ± 2 pA $pF^{-1}$ ($n = 7$) without Na-gluconate in the pipette solution. Statistical data are presented as mean ± s.e.m. **c**, Representative $I_{MCU}$ in control (left), in the presence of a constitutively active monomeric CaMKII (T287D mutant) in the patch pipette (middle), and in the presence of wild-type monomeric CaMKII previously activated (autophosphorylated) with $Ca^{2+}$/calmodulin (CaM) and $Mg^{2+}$/ATP (γ-thiol-ATP) (right) in the patch pipette. $I_{MCU}$ was elicited by a voltage ramp protocol (see panel **b**) in the presence of 0.2 and 105 mM $Ca^{2+}$. $I_{MCU}$ amplitude was monitored for up to 35 min after formation of the whole-mitoplast configuration as in Joiner *et al.*[7] (however, the calculated diffusion time[15] for the 35-kDa monomer of CaMKII from the pipette into the mitoplast is only ~25 s). Pipette solution contained (in mM): 150 Na-gluconate, 40 HEPES, 2 NaCl, 1.5 EGTA, tonicity 450 mmol per kg with sucrose, pH 7.2 with NaOH. The recombinant T287D and wild-type CaMKII were added to the control solution at 0.5 or 1 µM, in the presence of 2 mM $Na_2ATP$ and 3 mM $MgCl_2$. (Addition of ATP and $Mg^{2+}$ alone did not affect $I_{MCU}$.) **d**, Histogram showing average $I_{MCU}$ current densities obtained in the absence (black, control) or presence of T287D (red) or wild-type monomeric CaMKII pre-autophosphorylated with thiol-ATP (blue) in the pipette. Currents were measured in 0.2 and 105 mM $Ca^{2+}$ as described in **c**, and amplitudes were determined at 5 ms after stepping from 0 to –160 mV. $I_{MCU}$ densities were as follows: at bath 0.2 mM $Ca^{2+}$, 3.2 ± 0.3 pA $pF^{-1}$ ($n = 17$) in control, 3.2 ± 0.3 pA $pF^{-1}$ ($n = 14$) for T287D, and 3.0 ± 0.3 pA $pF^{-1}$ ($n = 8$) for autophosphorylated wild-type CaMKII; at bath 105 mM $Ca^{2+}$, 16.4 ± 0.5 pA $pF^{-1}$ ($n = 16$) in control, 17.9 ± 1.1 pA $pF^{-1}$ ($n = 11$) for T287D, and 16.2 ± 0.5 pA $pF^{-1}$ ($n = 5$) for autophosphorylated wild-type CaMKII. Statistical data are presented as mean ± s.e.m. **e**, Histogram showing average $I_{MCU}$ current densities in control (black) and in the presence of a constitutively active monomeric CaMKII (T287D mutant) in the patch pipette either alone (red) or with 1 µM CaM and 5–10 µM free $Ca^{2+}$ (green). $I_{MCU}$ densities were as follows: at bath 0.2 mM $Ca^{2+}$, 3.2 ± 0.3 pA $pF^{-1}$ ($n = 17$) in control, 3.2 ± 0.3 pA $pF^{-1}$ ($n = 14$) for T287D, and 2.8 ± 0.1 pA $pF^{-1}$ ($n = 5$) for T287D in the presence of 1 µM CaM and 5–10 µM free $Ca^{2+}$. Current amplitudes were measured at 5 ms after stepping from 0 to –160 mV. Statistical data are presented as mean ± s.e.m.



©2014 Macmillan Publishers Limited. All rights reserved

conditions used by Joiner *et al.*[7], and confirm that $I_{MCU}$ in cardiomyocytes is very small and is not directly regulated by CaMKII; thus, the currents presented by Joiner *et al.* do not appear to correspond to MCU, and there is no direct electrophysiological evidence that CaMKII regulates MCU. There is a Reply to this Brief Communication Arising by Joiner, M. A. *et al. Nature* **513,** http://dx.doi.org/10.1038/nature13627 (2014).

The main differences in the experimental conditions used by Joiner *et al.*[7] and in our previous study[3] were: the use of hypotonic shock to prepare mitoplasts (versus French press in our study), the presence of high $Na^+$ concentration in recording solutions (versus $Na^+$-free solutions), and the age of the mice (2–3 months versus 3–4 weeks).

Figure 1a shows mouse heart mitoplasts obtained by exposure of mitochondria to hypotonic shock. The measured average membrane capacitance ($C_m$) was $0.65 \pm 0.03$ pF ($\pm$ s.e.m., $n = 65$), which correlates well with $C_m$ measurements reported for heart mitoplasts obtained with French press[3], as well as with measurements of the inner mitochondrial membrane surface area using electron microscopy[8,9] and with estimated measurements of idealized cardiac mitochondria[10]. Therefore, the values reported by Joiner *et al.*[7] seem to be abnormally high (5−9 pF), indicating inaccuracy in monitoring $C_m$ leading to faulty values of $I_{MCU}$ densities throughout the paper.

We recorded $I_{MCU}$ from heart mitoplasts isolated by hypotonic shock with 150 mM Na-gluconate in the pipette and bath solutions (as in Joiner *et al.*[7]; Fig. 1b, left panel) and without $Na^+$ (conditions previously used by us[3]; Fig. 1b, middle panel). $I_{MCU}$ recorded in the presence of Na-gluconate was significantly smaller than in its absence (Fig. 1b). Our data support the observation that elevated $Na^+$ may regulate heart mitochondrial $Ca^{2+}$ concentration[11,12]. Notably, the whole-mitoplast $I_{MCU}$ was about two orders of magnitude lower than the current reported by Joiner *et al.*[7] (~2 pA at −160 mV in 0.2 mM $Ca^{2+}$ versus ~180 pA) and did not exhibit high fluctuations as expected for a small-conductance channel. Also, the current reported by Joiner *et al.*[7] was not inhibited by Ru360 in the same fashion as the $I_{MCU}$ (ref. 2). In 10 nM Ru360, $I_{MCU}$ shows no immediate inhibition upon stepping from 0 mV to −120 mV (ref. 2), and the inhibition develops slowly over time[2], whereas the current of Joiner *et al.*[7] was inhibited immediately upon stepping from 0 to −160 mV. All these observations indicate that Joiner *et al.*[7] did not record $I_{MCU}$. We suggest that either they did not record from inner mitochondrial membrane or the integrity of their mitoplasts was compromised.

Next, we tested whether $I_{MCU}$ is directly regulated by CaMKII, as claimed by Joiner *et al.*[7], who reported that addition of a constitutively active monomeric form of CaMKII (T287D mutant) to the patch pipette potentiated their currents. When we applied T287D mutant CaMKII, we failed to observe any functional change in $I_{MCU}$, either without (Fig. 1c, middle panel, and Fig. 1d) or with $Ca^{2+}$ plus calmodulin (Fig. 1e). We further verified these results using wild-type monomeric CaMKII pre-autophosphorylated with thiol-ATP to prevent de-autophosphorylation and again observed no change in $I_{MCU}$ (Fig. 1c, right panel, and Fig. 1d).

The noisy currents presented by Joiner *et al.*[7] do not appear to be carried by MCU, and their extremely high amplitude misrepresents the actual MCU activity in heart. Heart, with abundant mitochondria and frequently elevated cytosolic $Ca^{2+}$, has very low MCU current[3], which is probably critical for avoiding disruption of cytosolic $Ca^{2+}$ signalling and preventing mitochondrial $Ca^{2+}$ overload and cell death. Finally, our electrophysiological experiments with MCU currents did not indicate that MCU is regulated by CaMKII.

## Methods

Electrophysiological experiments were performed as in ref. 3. Recombinant δ-human monomeric CaMKII (1–317) was purified from baculovirus using an amino-terminal 6×-HN tag and Ni chromatography followed by gel filtration. Activity of recombinant CaMKII was measured in Na-gluconate pipette solution using the peptide substrate AC-2 (ref. 13). Constitutive activity (no $Ca^{2+}$/calmodulin) was undetectable for wild-type CaMKII and 4.6 μmol $min^{-1}$ $mg^{-1}$ for the T287D mutant. The $Ca^{2+}$/calmodulin stimulated activity of T287D CaMKII was 9.7 μmol $min^{-1}$ $mg^{-1}$. Wild-type CaMKII was autophosphorylated in γ-thiol-ATP to promote Thr 287 autophosphorylation, which allows CaMKII to be active without $Ca^{2+}$/calmodulin (that is, autonomous activity)[14]. The autonomous activity of wild-type CaMKII was 19.4 μmol $min^{-1}$ $mg^{-1}$ (~91% of the $Ca^{2+}$/calmodulin stimulated activity).

**Francesca Fieni[1], Derrick E. Johnson[2], Andy Hudmon[2] & Yuriy Kirichok[1]**
[1]Department of Physiology, University of California San Francisco, San Francisco, California 94158, USA.
email: yuriy.kirichok@ucsf.edu
[2]Department of Biochemistry and Molecular Biology, Stark Neuroscience Research Institute, Indiana University School of Medicine, Indianapolis, Indiana 46202, USA.

**Received 1 August 2013; accepted 17 June 2014.**

1. Rizzuto, R., Bernardi, P. & Pozzan, T. Mitochondria as all-round players of the calcium game. *J. Physiol.* **529,** 37–47 (2000).
2. Kirichok, Y., Krapivinsky, G. & Clapham, D. E. The mitochondrial calcium uniporter is a highly selective ion channel. *Nature* **427,** 360–364 (2004).
3. Fieni, F., Bae Lee, S., Jan, Y. N. & Kirichok, Y. Activity of the mitochondrial calcium uniporter varies greatly between tissues. *Nature Commun.* **3,** 1317 (2012).
4. Chaudhuri, D., Sancak, Y., Mootha, V. K. & Clapham, D. E. MCU encodes the pore conducting mitochondrial calcium currents. *eLife* **2,** e00704 (2013).
5. De Stefani, D., Raffaello, A., Teardo, E., Szabo, I. & Rizzuto, R. A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. *Nature* **476,** 336–340 (2011).
6. Baughman, J. M. *et al.* Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. *Nature* **476,** 341–345 (2011).
7. Joiner, M. A. *et al.* CaMKII determines mitochondrial stress responses in heart. *Nature* **491,** 269–273 (2012).
8. Page, E. Quantitative ultrastructural analysis in cardiac membrane physiology. *Am. J. Physiol.* **235,** C147–C158 (1978).
9. Smith, H. E. & Page, E. Morphometry of rat heart mitochondrial subcompartments and membranes: application to myocardial cell atrophy after hypophysectomy. *J. Ultrastruct. Res.* **55,** 31–41 (1976).
10. Williams, G. S., Boyman, L., Chikando, A. C., Khairallah, R. J. & Lederer, W. J. Mitochondrial calcium uptake. *Proc. Natl Acad. Sci. USA* **110,** 10479–10486 (2013).
11. O'Rourke, B. & Maack, C. The role of Na dysregulation in cardiac disease and how it impacts electrophysiology. *Drug Discov. Today Dis. Models* **4,** 207–217 (2007).
12. Maack, C. *et al.* Elevated cytosolic $Na^+$ decreases mitochondrial $Ca^{2+}$ uptake during excitation-contraction coupling and impairs energetic adaptation in cardiac myocytes. *Circ. Res.* **99,** 172–182 (2006).
13. Ashpole, N. M. & Hudmon, A. Excitotoxic neuroprotection and vulnerability with CaMKII inhibition. *Mol. Cell. Neurosci.* **46,** 720–730 (2011).
14. Rokita, A. G. & Anderson, M. E. New therapeutic targets in cardiology: arrhythmias and $Ca^{2+}$/calmodulin-dependent kinase II (CaMKII). *Circulation* **126,** 2125–2139 (2012).
15. Pusch, M. & Neher, E. Rates of diffusional exchange between small cells and a measuring patch pipette. *Pflugers Archiv.* **411,** 204–211 (1988).

**Author Contributions** F.F. and Y.K. conceived the project. F.F. performed electrophysiological experiments. D.E.J. and A.H. generated recombinant CAMKII and determined its activity under various conditions. All authors contributed to experimental design, discussed the results, and wrote the manuscript.

**Competing Financial Interests** Declared none.

**doi:**10.1038/nature13626



©2014 Macmillan Publishers Limited. All rights reserved

# Joiner *et al.* reply

REPLYING TO F. Fieni *et al. Nature* **513,** http://dx.doi.org/10.1038/nature13626 (2014)

In our Letter[1] identifying mitochondrial CaMKII as a crucial component of a $Ca^{2+}$-dependent process of heart disease, we used multiple methods to show that CaMKII modulates mitochondrial $Ca^{2+}$ homeostasis, as outlined below. First, we carried out electrophysiology of the mitochondrial calcium uniporter (MCU) current in mitoplasts. In our report[1] we did not claim to measure capacitance of the mitoplast separately from the total capacitance of the mitoplast and pipette. Although we concede that the approach of Fieni *et al.*[2] is preferable, we found that even after removing any correction for capacitance, dialysis with constitutively active CaMKII monomers increased MCU current whereas dialysis with catalytically dead CaMKII monomers did not.

Second, we identified candidate CaMKII sites on the MCU amino terminus (serine 57 and serine 92), and used a variety of approaches to provide evidence that both are functional and their phosphorylation by CaMKII increases MCU current. These approaches include transgenic mitochondrial-targeted expression of a highly selective CaMKII inhibitor (mtCaMKIIN), and dialysis of both constitutively active and catalytically inactive control CaMKII in cardiac mitoplasts, as well as in mitoplasts from HEK cells overexpressing wild-type MCU or a mutant form in which the two serines are replaced with alanines (S57A/S92A). We are uncertain why the dialysis by Fieni *et al.*[2], which used a constitutively active monomeric form of CaMKII, was ineffective in increasing MCU current, but given the tendency with which this kinase may lose activity we prefer a positive control experiment showing that the introduced CaMKII is active within a biological system.

Third, we measured a current from mitoplasts that we believe to be from MCU because: (1) it rectifies inwardly; (2) it displays greater $Na^{+}$ than $Ca^{2+}$ conductance; and (3) it is inhibited by Ru360 (albeit with a different kinetic signature than reported by Fieni *et al.*[2]). In addition to the voltage-clamp approach, we measured mitochondrial $Ca^{2+}$ entry with $Ca^{2+}$ green-5N dye and mitochondrial-targeted cameleon FRET, which also report on mitochondrial $Ca^{2+}$ entry via the MCU pathway (that is, MCU current), as confirmed by a recent report on MCU knockout mice[3]. Indeed, mitochondrial $Ca^{2+}$ uptake measured using $Ca^{2+}$ green-5N, intramitochondrial $Ca^{2+}$ sensitive probes and $Ca^{2+}$-induced mitochondrial swelling are absent in MCU knockout mice, indicating that these approaches are reporting on MCU current. These approaches, deployed in a variety of systems, corroborated our findings with voltage clamp and supported the role of CaMKII in facilitating mitochondrial $Ca^{2+}$ entry.

Since the time of our publication we have continued to study the role of CaMKII in mitochondria, including work to validate the Ser 57 and Ser 92 sites on MCU further. We note that our ongoing experiments support the concepts outlined in our original paper. The main finding in Joiner *et al.* was that CaMKII is present in cardiac mitochondria, where it participates in mitochondrial $Ca^{2+}$ homeostasis and contributes to pathological responses to stress, including myocardial infarction, ischaemia-reperfusion injury and catecholamine toxicity[1]. We stand by the findings in our report, and look forward to future studies that will shed light on the contribution of MCU, its regulatory subunits and signalling pathways to mitochondrial biology and disease.

**Mei-ling A. Joiner[1]†, Olha M. Koval[1], Jingdong Li[1]†, B. Julie He[1]†, Chantal Allamargot[2], Zhan Gao[1], Elizabeth D. Luczak[1]†, Duane D. Hall[1], Brian D. Fink[3], Biyi Chen[1], Jinying Yang[1], Steven A. Moore[4,5], Thomas D. Scholz[6], Stefan Strack[7], Peter J. Mohler[1]†, William I. Sivitz[1,3], Long-Sheng Song[1] & Mark E. Anderson[1,4]†**

[1]Department of Internal Medicine and Cardiovascular Center, Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242, USA.
email: mei-ling-joiner@uiowa.edu
[2]University of Iowa Central Microscopy Research Facility, Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242, USA.
[3]Iowa City Veterans Affairs Medical, Iowa City, Iowa 52246, USA.
[4]Department of Molecular Physiology and Biophysics, Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242, USA.
email: mark.anderson@jhmi.edu
[5]Department of Pathology, Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242, USA.
[6]Department of Pediatrics, Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242, USA.
[7]Department of Pharmacology, Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242, USA.
†Present addresses: Department of Molecular Physiology & Biophysics, Carver College of Medicine, University of Iowa, 51 Newton Road, Iowa City, Iowa 52242, USA (M.A.J.); The Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University, Columbus, Ohio 43210, USA (J.L., P.J.M.); Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611, USA (B.J.H.); Johns Hopkins University School of Medicine, 1830 East Monument Street, 9th Floor, Suite 9026, Baltimore, Maryland 21287, USA (E.D.L., M.E.A.).

1. Joiner, M. A. *et al.* CaMKII determines mitochondrial stress responses in heart. *Nature* **491,** 269–273 (2012).
2. Fieni, F., Johnson, D. E., Hudmon, A. & Kirichok, Y. Mitochondrial $Ca^{2+}$ uniporter and CaMKII in heart. *Nature* **513,** http://dx.doi.org/10.1038/nature13626 (2014).
3. Pan, X. *et al.* The physiological role of mitochondrial calcium revealed by mice lacking the mitochondrial calcium uniporter. *Nature Cell Biol.* **15,** 1464–1472 (2013).

**doi:**10.1038/nature13627



©2014 Macmillan Publishers Limited. All rights reserved