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Mitochondrial Ca^{2+} Uniporter and CaMKII in heart

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Abstract

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]. Also, 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 conditions used by Joiner et al. We confirmed that I_{MCU} in cardiomyocytes is very small and showed that it is not directly regulated by CaMKII. Thus the currents presented by Joiner et al. do not correspond to MCU, and there is no direct electrophysiological evidence that CaMKII regulates MCU.

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 (vs. French Press in our study), the presence of high Na^+ concentration in recording solutions (vs. Na^+-free solutions), and the age of the mice (2–3 months vs. 3–4 weeks).

Fig. 1a shows mouse heart mitoplasts obtained by exposure of mitochondria to hypotonic shock. The measured average membrane capacitance (C_m) was 0.65±0.03 pF (±SEM, 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 EM[8,9] and with estimated measurements of idealized cardiac mitochondria[10]. Therefore, the values reported by Joiner et al. are 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 NaGluconate in the pipette and bath solutions (as in Joiner et al., Fig. 1b, left panel) and without Na^+ (conditions previously used by us[3], Fig. 1b, middle panel). Interestingly, I_{MCU}

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recorded in the presence of NaGluconate was significantly smaller than in its absence (Fig. 1b). Our data support the observation that elevated Na^+ may regulate heart mitochondrial $[Ca^{2+}]$[11,12]. Importantly, the whole-mitoplast I_{MCU} was about two orders of magnitude lower than the current reported by Joiner et al. (∼2 pA at -160 mV in 0.2 mM Ca^{2+} vs. ∼180 pA) and did not exhibit high fluctuations as expected for a small-conductance channel. Also, the current reported by Joiner et al. was not inhibited by Ru360 in the same fashion as the I_{MCU}[2]. In 10 nM Ru360, I_{MCU} shows no immediate inhibition upon stepping from 0 mV to -120 mV[2], and the inhibition develops slowly over time[2], whereas the current of Joiner et al. was inhibited immediately upon stepping from 0 to -160 mV. All these observations indicate that Joiner et al. 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., 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, we failed to observe any functional change in I_{MCU}, either without (Fig 1c middle panel, and d) 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 d).

In conclusion, the noisy currents presented by Joiner et al. are not 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 likely critical for avoiding disruption of cytosolic Ca^{2+} signaling 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 Fieni et al[3]. Recombinant δ–human monomeric CaMKII (1-137) was purified from baculovirus using an N-terminal 6X-HN tag and Ni chromatography followed by gel filtration. Activity of recombinant CaMKII was measured in NaGluconate pipette solution using the peptide substrate AC-2[13]. Constitutive activity (no Ca^{2+} /calmodulin) was undetectable for wild-type CaMKII and 4.6 μmol/min/mg for T287D. The Ca^{2+}/calmodulin stimulated activity of T287D CaMKII was 9.7 μmol/min/mg. Wild-type CaMKII was autophosphorylated in γ-thiol-ATP to promote Thr287 autophosphorylation, which allows CaMKII to be active without Ca^{2+}/calmodulin (i.e., autonomous activity)[14]. The autonomous activity of wild-type CaMKII was 19.4 μmol/min/mg (∼91% of the Ca^{2+}/calmodulin stimulated activity).

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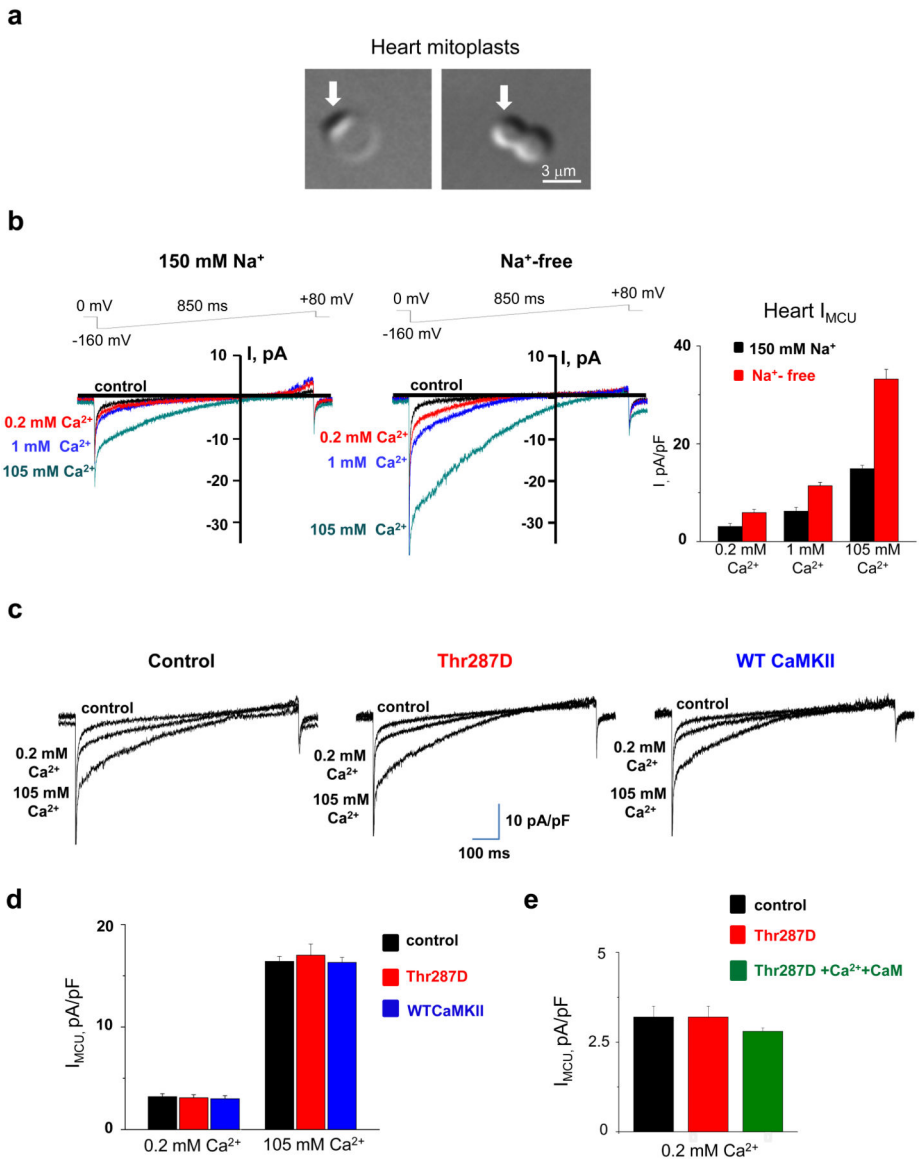

Fig. 1. Heart MCU current and CaMKII

(a) Transmitted image of heart mitoplasts obtained by exposure of mitochondria to 5-minute hypotonic shock. Both round (*left panel*) and figure 8-shaped (*right panel*) mitoplasts were present in this preparation and used for electrophysiological experiments. Arrows indicate remnants of the outer mitochondrial membrane. Note that 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 panel*) or absence (*middle panel*) of 150 mM NaGluconate 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. Note that 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 panel* and Fieni et al[3]). Pipette solution, in mM: 150 NaGluconate, 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 NaGluconate, 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. R*ight panel*, histogram representing average MCU current densities (I_{MCU} normalized to the C_m) obtained in the presence (black) or absence (red) of 150 mM NaGluconate 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 (n = 8) with 150 NaGluconate in recording solutions and 6±0.7 pA/pF without NaGluconate in recording solutions; at bath 1 mM Ca^{2+}, 6.2±0.7 pA/pF (n = 9) with NaGluconate and 11.4±0.7 pA/pF (n=6) without NaGluc; at bath 105 mM Ca^{2+}, 14.2±0.7 pA/pF (n = 12) with NaGluconate and 33.2±2 pA/pF (n=7) without NaGluconate in the pipette solution. Statistical data are presented as mean ± SEM. **(c)** Representative I_{MCU} in control (*left panel*), in the presence of a constitutively active monomeric CaMKII (Thr287D mutant) in the patch pipette (middle panel), 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 panel*) 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. (However, the calculated diffusion time[15] for the 35-kDa monomer of CaMKII from the pipette into the mitoplast is only ∼25 seconds.) Pipette solution contained, in mM: 150 NaGluconate, 40 HEPES, 2 NaCl, 1.5 EGTA, tonicity 450 mmol per kg with sucrose, pH 7.2 with NaOH. The recombinant Thr287D 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 Thr287D (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 (n=17) in control, 3.2±0.3 pA/pF (n=14) for Thr287D, and 3.0±0.3 pA/pF (n=8) for autophosphorylated wild-type CaMKII; at bath 105 mM Ca^{2+}, 16.4±0.5 pA/pF (n=16) in control, 17.9±1.1 pA/pF (n=11) for Thr287D, and 16.2±0.5 pA/pF (n=5) for autophosphorylated wild-type CaMKII. Statistical data are presented as mean ± SEM. **(e)** Histogram showing average I_{MCU} current densities in control (black) and in the presence of a constitutively active monomeric CaMKII (Thr287D 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 (n=17) in control, 3.2±0.3 pA/pF (n=14) for Thr287D, and 2.8±0.1 pA/pF (n=5) for Thr287D 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 ± SEM.