The mitochondrial uniporter controls fight or flight heart rate increases

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Heart rate increases are a fundamental adaptation to physiological stress, while inappropriate heart rate increases are resistant to current therapies. However, the metabolic mechanisms driving heart rate acceleration in cardiac pacemaker cells remain incompletely understood. The mitochondrial calcium uniporter (MCU) facilitates calcium entry into the mitochondrial matrix to stimulate metabolism. We developed mice with myocardial MCU inhibition by transgenic expression of a dominant-negative (DN) MCU. Here, we show that DN-MCU mice had normal resting heart rates but were incapable of physiological fight or flight heart rate acceleration. We found that MCU function was essential for rapidly increasing mitochondrial calcium in pacemaker cells and that MCU-enhanced oxidative phosphorylation was required to accelerate reloading of an intracellular calcium compartment before each heartbeat. Our findings show that MCU is necessary for complete physiological heart rate acceleration and suggest that MCU inhibition could reduce inappropriate heart rate increases without affecting resting heart rate.
Catecholamine agonists trigger physiological flight or fight increases in heart rate but the metabolic pathway(s) supplying adenosine triphosphate (ATP) for increasing heart rate are incompletely understood1,2. Cardiac pacemaker cells drive heart rate acceleration, at least in part, by augmenting energy-dependent flux of Ca$^{2+}$ through an intracellular, sarcoplasmic reticulum (SR), storage compartment3. SR Ca$^{2+}$ release triggers pacemaker cell membrane depolarization, leading to action potential initiation that triggers each heart beat4. Mitochondrial Ca$^{2+}$ entry through the mitochondrial Ca$^{2+}$ uniporter (MCU) can stimulate increased ATP production by enhancing the activity of dehydrogenases in the mitochondrial matrix that supply NADH for electron transport5,6. The recent discovery of the gene encoding the MCU protein Ca$^{2+}$-permeation pore$^{7,8}$ allowed us to test the potential role of MCU-mediated mitochondrial Ca$^{2+}$ entry as a pathway for increasing ATP production to fuel heart rate increases. We developed new tools and approaches for studying the metabolic role of the MCU in cardiac pacing, including surgical gene transfer to pacemaker cells and transgenic mice with myocardial and pacemaker cell targeted expression of a dominant-negative (DN) MCU with pore domain mutations that prevented rapid, MCU-mediated mitochondrial Ca$^{2+}$ entry. Here, we show that Ca$^{2+}$ entry through the MCU is essential for telegraphing enhanced metabolic demand to pacemaker cell mitochondria and promoting oxidative phosphorylation. We found that isoproterenol (ISO) stimulates oxidative phosphorylation by the MCU pathway in cardiac pacemaker cells to fuel the activity of the sarcoplasmic-endoplasmic reticulum Ca$^{2+}$ ATPase (SERCA2a), which is required for reloading SR Ca$^{2+}$ stores and sustaining fight or flight heart rate increases. Inhibition of mitochondrial Ca$^{2+}$ entry prevented increased oxidative phosphorylation, enhanced SERCA2a activity and physiological rate responses in cardiac pacemaker cells exposed to ISO. Dialysis of cardiac pacemaker cells with exogenous ATP rescued the flight or flight response to ISO despite MCU inhibition but ATP dialysis was ineffective after SERCA2a inhibition, by expression of a super-inhibitory phospholamban (PLN) mutant or thapsigargin, identifying SERCA2a as a critical control point downstream of MCU for heart rate increases and a preferential sink for mitochondrially-sourced ATP. Isolated hearts from wild-type mice with pacemaker-targeted DN-MCU gene therapy were resistant to rate increases by ISO. We found selectively obtunded ISO triggered rate increases in isolated pacemaker cells, in excised Langendorff-perfused hearts from wild-type mice with DN-MCU pacemaker-targeted gene therapy and in vivo in DN-MCU transgenic mice. Furthermore, DN-MCU transgenic mice showed reduced heart rates in response to spontaneous activity compared with wild-type littermate controls. In contrast to the profound loss of heart rate acceleration by MCU inhibition, unstimulated heart rates and autonomous In contrast to the profound loss of heart rate acceleration by MCU inhibition, unstimulated heart rates and autonomous

**Results**

**The MCU mediates rate increases in pacemaker cells.** Isolated cardiac sinoatrial nodal (SAN) pacemaker cells spontaneously generate action potentials under basal conditions, in the absence of catecholamine stimulation. The rate of action potential initiation is increased with ISO, a catecholamine β-adrenergic receptor agonist, in a concentration-dependent manner (Fig. 1a–d)$^{10}$. We found that SAN cells dialysed with Ru360 (5 μM), an MCU antagonist, had significantly reduced action potential frequency increases to ISO (Fig. 1b,d) compared with SAN cells without Ru360 (Fig. 1a,d). The inhibitory effect of Ru360 on SAN cell action potential frequency responses to ISO was reversed by co-dialysis with ATP (4 mM), a concentration present in heart cells (Fig. 1c,d)$^{11}$. Co-dialysis with 1 and 2 mM ATP pipette solutions was inadequate to rescue ISO-mediated rate increases while 8 mM ATP did not result in greater rate responses than 4 mM ATP (Supplementary Fig. 1a–e), suggesting flight or fight SAN responses operate within an [ATP] threshold $>2$ and $<4$ mM. In contrast, neither Ru360 nor ATP had any effect on basal SAN cell rates and ATP dialysis had no effect on SAN rate responses to ISO in the absence of Ru360 (Supplementary Fig. 1a,b), indicating that basal heart rate was independent of an MCU pathway and that exogenous ATP did not affect Ru360-independent cellular processes important for accelerating heart rate. We next corroborated our results with Ru360 dialysis by infecting SAN cells with adenosine encoding a DN-MCU, containing pore domain charge reversal mutations that prevent MCU-mediated mitochondrial Ca$^{2+}$ entry (Fig. 1e–h)$^{7,8}$. Both Ru360 and DN-MCU were similarly effective at preventing mitochondrial Ca$^{2+}$ entry (Supplementary Fig. 2) and, like intracellular dialysis with Ru360, DN-MCU expression in SAN cells interfered with ISO-induced rate increases (Fig. 1f,h). The DN-MCU-dependent loss of the ISO rate response was rescued by co-dialysis with ATP (Fig. 1g,h), mirroring findings with MCU inhibition by Ru360 (Fig. 1c,d). Cultured SAN cells, with and without DN-MCU expression, had slower basal spontaneous rates than freshly isolated counterparts (baseline in Fig. 1d,h) but nevertheless exhibited significant rate increases over baseline in response to ISO (Fig. 1g,i). We considered the possibility that the mitochondrial Na$^{+}$/Ca$^{2+}$ exchanger, a MCU-independent pathway for mitochondrial Ca$^{2+}$ efflux, could also contribute to SAN rate increases$^{12}$ by testing SAN responses to CGP-37157 (1 μM), a Na$^{+}$/Ca$^{2+}$ exchanger antagonist. After CGP-37157, we measured a 1.2±0.6% increase in WT SAN cells (n=8) and 0.8±2.6% increase in DN-MCU SAN cells (n=3, P=0.88), suggesting that the mitochondrial Na$^{+}$/Ca$^{2+}$ exchanger did not affect basal heart rate. After ISO (1 μM), we measured a 79.8±5.8% (n=4) increase from baseline rate in WT SAN cells and 32.0±11.1% increase from baseline (n=4) rate in DN-MCU SAN cells (P<0.01), similar to ISO-mediated increases in the absence of CGP-37157 (Fig. 1i). These findings suggested that the mitochondrial Na$^{+}$/Ca$^{2+}$ exchanger was unlikely to participate in SAN rate responses, consistent with other recent findings$^{13}$. The rescue of physiological responses to ISO after MCU inhibition by ATP dialysis suggested that MCU activity was an upstream event required to supply adequate ATP for increasing heart rate.

**MCU inhibition impairs heart rate acceleration.** The selective control of pacemaker cell rate increases by MCU inhibition suggested that the MCU could be a novel target for controlling heart rate increases without slowing resting heart rates. To test this concept in hearts, we used a targeted gene-painting approach to deliver adenovirus expressing DN-MCU or eGFP to the SAN in vivo$^{14}$. One week after SAN gene painting, we verified SAN targeted gene expression (Fig. 2a,b) and measured rate responses to ISO in excised, Langendorff-perfused hearts (Fig. 2c–f). We
Figure 1 | SAN cell rate response to ISO is impaired by Ru360 or DN-MCU and rescued by ATP. (a–c) Example action potential (AP) tracings recorded under basal (left) and ISO-stimulated (right) conditions from freshly isolated SAN cells. The horizontal line marks 0 mV. Scale bars are 100 ms horizontal and 20 mV vertical. (d) Summary dose–response data for SAN cell AP rate responses to ISO. (e–g) Example AP tracings recorded under basal (left) and ISO-stimulated (right) conditions from cultured SAN cells. The horizontal line marks 0 mV. Scale bars are the same as in (a–c). (h) Summary dose–response data for cultured SAN cell AP rate responses to ISO. (i,j) Percentage rate increases over baseline after ISO in isolated SAN cells. (i) Freshly isolated SAN cells (data calculated from d). (j) Cultured SAN cells (data calculated from h). *P < 0.05, **P < 0.01, ***P < 0.001, one-way analysis of variance, n = 10–15 per group in d, n = 5–9 per group in h. Error bars indicate s.e.m.

Figure 2 | DN-MCU gene painting impairs ISO rate response in isolated hearts. (a,b) Immunofluorescent staining of (a) control right atrium and (b) gene-painted SAN showing DAPI (blue) and HCN4 (red) to identify SAN, and Myc epitope tag (green) for DN-MCU-Myc expression. Scale bar, 200 μm. (c,d) Example ex vivo ECG tracings recorded under basal (left) and ISO-stimulated (right) conditions from gene-painted hearts. Scale bar, 100 ms. (e) Summary dose–response data for ex vivo heart rate response to ISO. (f) Percentage rate increases over baseline after ISO in gene-painted isolated, Langendorff-perfused hearts (data calculated from e). ***P < 0.001, unpaired Student’s t-test, n = 22–24 per group in e. Error bars indicate s.e.m.
Figure 3 | Characterization of the DN-MCU transgenic mouse. (a) Schematic of the α-myosin heavy chain promoter-driven transgenic DN-MCU construct. (b) Western blots for Myc-tagged DN-MCU protein from heart (H), liver (L) and skeletal muscle (S) lysates (WT, DN-MCU). (c) Images show 10 μm sections through the SAN area. Anti-Myc (green) antibody identified cells expressing DN-MCU and the SAN was identified by anti-a-MHC antibody (red). Scale bar, 25 μm. (d) Data show ATP concentrations measured in freshly isolated atrial tissue from DN-MCU and littermate WT mice. ***P<0.0001, unpaired Student’s t-test. (e) Representative Ca²⁺ uptake tracings of three similar experiments in permeabilized cardiac myocytes from DN-MCU and littermate WT mice (as in Supplementary Fig. 2). Arrows repress the addition of Ca²⁺ (100 μM). (f) Example in vivo ECG tracings recorded under basal (left) and ISO-stimulated (right) conditions from DN-MCU and littermate WT mice. Scale bar, 100 ms. (g) Summary echocardiographic data showing mean ± standard deviation (WT, n = 21; DN-MCU, n = 20). NS was identified in any parameter: heart rate (HR), ejection fraction (EF), left ventricular mass (LV mass), end diastolic volume (EDV), end systolic volume (ESV), stroke volume (SV) and cardiac output (CO). (h) Percentage rate increases over baseline after ISO in in vivo ECG recordings (data calculated from j). (i) In vivo heart rate response to activity was reduced in DN-MCU mice, n = 8–11 per group. **P<0.01, ***P<0.001, unpaired Student’s t-test. (j) Percentage rate increases over baseline in in vivo telemetry ECG recording with peak activity (data calculated from l). (m) In vivo heart rate response to ISO stimulation was reduced by DN-MCU expression in DN-MCU mice, n = 11–28 per group. Error bars indicate s.e.m. *P<0.05, **P<0.01, ***P<0.001, unpaired Student’s t-test. (o) Percentage rate increases over baseline after ISO in in vivo telemetry ECG recording with ISO (data calculated from n).

found DN-MCU SAN gene painting significantly and selectively reduced heart rate increases to ISO without affecting spontaneous heart rates in the absence of ISO. We next developed transgenic mice with myocardial DN-MCU expression (Fig. 3a,b and Supplementary Fig. 3) to test the role of MCU in heart rate acceleration in vivo. The DN-MCU mice had hearts with normal chamber size and function (Fig. 3c), detectable SAN DN-MCU expression (Fig. 3d), reduced ATP content (Fig. 3e) and complete
loss of mitochondrial Ca\(^{2+}\) uptake in myocardial cells (Fig. 3f,g). DN-MCU hearts showed increased Mcu mRNA, as expected, and reduced mRNA expression for auxiliary MCU regulatory proteins (Supplementary Fig. 4), possibly consistent with compensatory transcriptional reprogramming in response to the loss of functional MCU. The reduced fight or flight response in DN-MCU hearts was selective for catecholamine agonist stimulation because DN-MCU and wild-type SAN cells had similar rate responses to BayK 8644 (1 μM), a voltage-gated Ca\(^{2+}\) channel agonist capable of accelerating heart rate independent of ISO. In the presence of BayK 8644 DN-MCU SAN cells (n = 5) exhibited 69.9 ± 10.9% rate increases over baseline, similar to published responses in wild-type SAN cells\(^{15}\). During echocardiography measurements, lightly sedated DN-MCU mice exhibited resting heart rates similar to wild-type littermate controls (Fig. 3c). Lightly and restrained DN-MCU and wild-type mice during cutaneous electrocardiogram (ECG) recording showed similar resting heart rates (Fig. 3h–j), but reduced ISO-stimulated heart rate increases (Fig. 3k). DN-MCU mice had significantly prolonged P-waves, PQ and PR intervals but similar QRS and QT interval durations compared with WT controls, suggesting that loss of MCU current slows atrial and atrioventricular conduction velocity without affecting conduction velocity in the distal conduction system or in ventricular myocardium (Supplementary Table 1). Finally, we measured heart rates in unrestrained and unsedated mice with surgically implanted ECG and activity telemeters. DN-MCU mice showed modest but significant reductions in basal heart rate compared with wild-type controls and these differences were enhanced by activity (Fig. 3l,m) and ISO (Fig. 3n,o). Taken together, these data showed heart rate responses to spontaneous activity and ISO required MCU, whereas basal heart rates were independent of MCU, indicating MCU inhibition could selectively prevent heart rate increases in vivo.

MCU enables rapid refilling of SR Ca\(^{2+}\) in pacemaker cells. Catecholamine stimulation increases heart rate by enhancing SAN cell membrane inward current and shortening the time between action potential firing\(^{16}\). Release of intracellular Ca\(^{2+}\) from the SR provides the electrochemical driving force for the cell membrane Na\(^{+}\)/Ca\(^{2+}\) exchanger inward current (I\(_{\text{Ncx}}\)) in SAN cells\(^{17}\). Elimination of SR Ca\(^{2+}\) release by the toxin ryanodine or SERCA2a inhibition by thapsigargin (Supplementary Fig. 5) significantly reduced the SAN response to ISO, findings that demonstrate the required connection between SR Ca\(^{2+}\) release and physiological SAN cell acceleration\(^{18}\). To further test the apparent connection between MCU activity and SR Ca\(^{2+}\) flux, we performed confocal line-scan measurements on SAN cells isolated from DN-MCU and wild-type control mice (Fig. 4). DN-MCU SAN cells had significantly fewer diastolic Ca\(^{2+}\) release events and reduced SR Ca\(^{2+}\) content after ISO stimulation compared to wild-type controls, suggesting that impaired fight or flight responses by MCU inhibition occurred because of reduced SR Ca\(^{2+}\) flux during physiological stress. SERCA2a activity requires ATP to pump cytoplasmic Ca\(^{2+}\) to the SR lumen\(^{19}\), so we measured the rate of decay of the cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{cyto}}\)) using a fluorescent indicator (Fura 2 AM, 0.1 μM) to test if SERCA2a was a sink for ATP produced by an Ru360-sensitive process. ISO significantly increased the rate of decline in [Ca\(^{2+}\)]\(_{\text{cyto}}\) compared with SAN cells in control bath solution, reflecting enhanced activity of SERCA2a (Fig. 5a)\(^{20}\). Ru360 dialysis slowed the decline in [Ca\(^{2+}\)]\(_{\text{cyto}}\) after ISO (Fig. 5b), while co-dialysis of ATP (4 mM)...
with Ru360 restored the rate of $[\text{Ca}^{2+}]_{\text{cyto}}$ decline to ISO-stimulated values present in the absence of Ru360 (Fig. 5c,d). However, Ru360 did not slow the decline in $[\text{Ca}^{2+}]_{\text{cyto}}$ in the absence of ISO stimulation (Fig. 5e), consistent with the lack of effect of Ru360 on SERCA2a activity or basal SAN cell action potential frequency. In contrast to the effect of ATP dialysis on SAN cells exposed to Ru360, ATP dialysis did not significantly increase the rate of $[\text{Ca}^{2+}]_{\text{cyto}}$ decline (Fig. 5f-h) or SAN action potential frequency (Fig. 5i) in SAN cells isolated from mice expressing a super-inhibitory mutant form of phospholamban (N27A)\(^{21}\) that constrains SERCA2a despite ISO stimulation. These results show that ISO increases SAN cell rates by actions that require MCU and SERCA2a. We considered the possibility that MCU inhibition was somehow affecting the ability of ISO to enhance phospholamban phosphorylation, which reduces the inhibitory actions of phospholamban on SERCA2a\(^{22}\). We found that atrial tissues from DN-MCU and wild-type littermates had similar increases in phospholamban phosphorylation after ISO (Supplementary Fig. 6), suggesting that MCU inhibition did not interfere with SERCA2a activity by actions on phospholamban, nor did DN-MCU expression promiscuously affect downstream signalling actions of ISO. We interpreted the rescue of ISO responses by exogenous ATP after the elimination of MCU-mediated $\text{Ca}^{2+}$ entry but not after SERCA2a inhibition to suggest that MCU contributes to ATP synthesis targeted for SERCA2a activation during physiological stress.

**MCU is not a global effector of pacemaker currents.** Because heart rate is responsive to multiple ionic currents\(^{16}\), we next asked if the MCU pathway affected the ATP and 3′,5′-cyclic adenosine monophosphate (cAMP)-dependent cell membrane ion channel (HCN4) inward current (I\(_{\text{f}}\))\(^{23}\) an ionic current known to participate in SAN cell automaticity. We found that I\(_{\text{f}}\) responses to ISO in isolated SAN cells were not reduced by Ru360 dialysis (Fig. 6a,b). The lack of effect of Ru360 on I\(_{\text{f}}\) suggested that Ru360 did not reduce cAMP nor ATP availability globally in SAN cells below a threshold necessary to increase I\(_{\text{f}}\). To test this concept further, we measured the maximum diastolic cell membrane potential, which is primarily determined by activity of the Na\(^+\)/K\(^+\) ATPase\(^{16}\). MCU inhibition by Ru360 dialysis or by transgenic expression of DN-MCU did not affect the maximum diastolic membrane potential in isolated SAN cells (Fig. 6c). We also measured Ca\(_{\text{i}}\) L-type $\text{Ca}^{2+}$ current (I\(_{\text{Ca,L}}\)), an SAN cell membrane inward current enhanced by ISO through ATP-mediated phosphorylation\(^{24}\). Similar to our findings with I\(_{\text{f}}\), ISO-induced I\(_{\text{Ca,L}}\) increases were not impaired by Ru360 (Fig. 6d,e). These findings were consistent with a model where selective loss of heart rate acceleration after ISO by Ru360 was primarily or exclusively related to actions on SERCA2a.

**MCU is required for ISO to increase NADH.** Mitochondrial $\text{Ca}^{2+}$ entry increases oxidative phosphorylation by enhancing the activity of key mitochondrial dehydrogenases to provide NADH/NADPH-reducing equivalents required for ATP synthesis\(^{25}\). This mechanism is activated when cellular $\text{Ca}^{2+}$ enters the inner mitochondrial membrane space from the cytosol through the MCU pathway\(^{26}\). We first asked whether mitochondrial $\text{Ca}^{2+}$ entry was critical for oxidative phosphorylation-dependent ATP synthesis in SAN pacemaker cells. We infected cultured mouse SAN cells with adenovirus encoding mt-pericam\(^{27}\), a circularly permutated $\text{Ca}^{2+}$-sensitive fluorescent protein, to measure mitochondrial $\text{Ca}^{2+}$ concentration ([Ca\(_{\text{mito}}\)]). The mt-pericam expression was localized to mitochondria in adenosine infected SAN pacemaker cells, based on co-localization with MitoTracker Orange (Fig. 7a). ISO caused...
Figure 6 | $I_f$ and $I_{Ca}$ ISO responses and maximum diastolic cell membrane potential are MCU independent. (a) Representative $I_f$ current recordings in response to voltage clamp commands (top panel) from an SAN cell without Ru360 (middle panel) and an Ru360-dialysed SAN cell (lower panel) before (left) and after ISO (right). (b) Summary current-voltage relationship for $I_f$ from groups shown in a, $n=4$ SAN cells without Ru360 (−Ru360) and $n=5$ Ru360-dialysed cells (+Ru360). NS for $I_f$ current density in Ru360-dialysed compared with SAN cells without Ru360 before and after ISO, unpaired Student’s t-test. (c) Maximum diastolic potential of spontaneous action potentials was not altered by MCU inhibition. $n=12$ per group. (d) Representative $I_{Ca}$ current recordings in response to voltage clamp commands (top panel) from an SAN cell without Ru360 (-Ru360, middle panel) and an Ru360-dialysed SAN cell (+ Ru360, lower panel) before (black trace) and after ISO (blue trace). (e) Summary current-voltage relationship for $I_{Ca}$ from groups shown above. $n=6$–8 per group. NS for $I_{Ca}$ current density in +Ru360 compared with −Ru360 cells before and after ISO, unpaired Student’s t-test. Error bars indicate s.e.m.

Discussion

Our data provide new mechanistic understanding into the fight or flight response to physiological stress by showing that heart rate increases rely on the MCU in cardiac pacemaker cells. In contrast, basal rates do require SERCA activity but are independent of MCU, suggesting that availability of MCU-independent ATP production is sufficient to sustain heart rates in the absence of extreme physiological stress. Oxidative phosphorylation is enhanced by $[\text{Ca}^{2+}]_{\text{mito}}$ which is required to generate ATP that fuels SERCA2a activity under extreme physiological stress. Our findings show that $\text{Ca}^{2+}$-sensitive homeostatic mechanisms in pacemaker cells form the framework for fight or flight heart rate increases but do not exclude additional modulation of heart rate by other $\text{Ca}^{2+}$ sensitive signals or by $\text{Ca}^{2+}$-independent ionic currents. The MCU metabolic pathway appears optimized to generate heart rate increases during episodes of high energy demand that are signalled by catecholamines. Our data are consistent with earlier work showing that mitochondrial $\text{Ca}^{2+}$ is required to optimize refilling of intracellular $\text{Ca}^{2+}$ stores by SERCA2a and where ATP dialysis (3 mM) recovered $\text{Ca}^{2+}$ sequestration by intracellular stores after the addition of mitochondrial toxins. Our findings provide insight into recent work showing MCU knockout selectively impairs high workload activity in striated muscle. However, our data show that basal...
Figure 7 | ISO increases SAN cell mitochondrial Ca\(^{2+}\). (a) Adenovirus infected SAN cells expressing mt-pericam co-localized with MitoTracker (scale bar, 10 \(\mu\)m). (b) The mt-pericam fluorescence signal was increased by ISO (100 nM) stimulation and Ru360 dialysis prevented this increase (scale bar, 10 \(\mu\)m). (c) Example tracings show ISO increases in mitochondrial \([Ca^{2+}]_{\text{mito}}\) were prevented by Ru360 dialysis (scale bar, 100 s). (d) Summary data for \([Ca^{2+}]_{\text{mito}}\) responses to ISO. ***P < 0.001, n = 14-15 per group, unpaired Student’s t-test. Error bars indicate s.e.m.

Figure 8 | Ru360 prevents ISO-stimulated increases in NADH. (a) Example of NADH fluorescence responses to ISO (100 nM) in control (–Ru360) and Ru360 dialysed SAN cells. Scale bar, 100 s. (b) Summary data for NADH fluorescence responses to ISO. ***P < 0.001, n = 7-18 per group, unpaired Student’s t-test. Error bars indicate s.e.m. (c) Proposed mechanism for MCU-enhancing SAN responses to ISO. β-AR, beta adrenergic receptor; CaV, voltage-dependent Ca\(^{2+}\) channels; DN-MCU, dominant-negative MCU; HCN, hyperpolarization-activated cyclic nucleotide-gated channels; MCU, mitochondrial calcium uniporter; N27A, super-inhibitory phospholamban; NCX, Na\(^+\)/Ca\(^{2+}\) exchanger; PLN, phospholamban; SERCA, sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase; SR, sarcoplasmic reticulum; TG, thapsigargin.
pacemaker cell activity is uncoupled from MCU-dependent ATP production. Because the relationship between MCU and SERCA2a in pacemaker cells appears positioned to selectively enable heart rate acceleration, future therapies targeting MCU or SERCA2a in pacemaker cells could provide a means to fine tune heart rates by preventing excessive heart rates without reducing resting heart rates.

Methods

All the experiments were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee (PSI Animal Welfare Assurance, A3021-01).

SAN cell isolation and electrophysiological recordings. Isolation of single SAN cells from mice was performed according to previously published method29,34 with minor modifications. Mice (6–8 weeks, females and males) were administered an intraperitoneal injection of avertin (20 mg\% with minor modifications. Mice (6–8 weeks, females and males) were administered

NADH and mitochondrial Ca2+ measurements. The autofluorescence of endogenous NADH, which derives primarily from mitochondria40,41, was measured as previously described38. In brief, NADH was excited at 350 nm and its fluorescence was recorded at 460 nm (ET460/50m and T400LP, Chroma). We normalized NADH level with FCCP as 0%, Rotenone-induced NADH change as 100%. The baseline level of NADH was 22% ± 3 (n = 15) of the rotenone-induced maximal value.

Intracellular Ca2+ transients. Cytosolic Ca2+ transients were recorded from Fura-2–loaded cells, excited at wavelengths of 340 and 380 nm, and imaged with a 510-nm long-pass filter. Single isolated SAN cells were loaded with 0.1 μM Fura-2 AM for 20 min and then perfused for 20 min to de-esterify the Fura-2 AM in normal Tyrode’s solution. After placement on a recording chamber, the cells were perfused in normal Tyrode’s solution at 36 °C ± 0.5. Spindle-shaped, spontaneously beating cells were chosen for the experiments. Action potential recording was performed simultaneously.

Ex vivo Langendorff-perfused heart rate recordings. ECG recording from Langendorff-perfused hearts was performed as described37. Briefly, excised hearts from 7–8 week female and male mice were rapidly mounted on a modified Langendorff apparatus (HSE-HA perfusion systems, Harvard Apparatus, Holliston, Mass) for retrograde aortic perfusion at a constant pressure of 80 mm Hg with oxygenated (95% O2, 5% CO2) Krebs–Henseleit solution consisting of (mM) 25.0 NaHCO3, 118.5 NaCl, 4.0 KCl, 1.2 MgSO4, 1.2 Na2HPO4, 1.5 CaCl2 and 11.2 glucose, with pH equilibrated to 7.4. Each perfused heart was immersed in a water-jacketed bath and was maintained at 36 °C. ECG measurements from the intact heart were continuously recorded with Ag+–AgCl electrodes, which were positioned around the heart in an approximate Einthoven configuration. After the heart was allowed to stabilize for 15 min, different concentrations of isoproterenol were added to the perfusate.

Immunofluorescence staining. The SAN area was dissected from freshly isolated hearts for comparing transgenic DN-MCU mice with WT or from Langendorff-perfused hearts for localizing SAN gene painted tissue. The SAN tissue was imbedded in OCT tissue freezing media and cooled over liquid nitrogen before being stored at −80 °C. 10 μM serial sections were taken through the tissue. All sections were slowly brought to −20 °C to room temperature in 4% paraformaldehyde. Sections were washed with ice cold PBS, permeabilized (0.1% Triton X-100, 0.1% sodium citrate in PBS) and blocked (3% fish gelatin, 2 mg ml−1 BSA, 0.1% Triton X-100 in PBS). HCN4 (Abcam) (1:200) and Myc epitope tag (Rockland) (1:200) antibodies were incubated with samples overnight at 4 °C. Samples were washed in blocking buffer and fluorochrome conjugated secondary antibodies (1:500) were added overnight. Genetic pacemaker cells were treated with DAPI and imaged on an EVOS FL Auto microscope. DN-MCU transgenic samples and WT sections were images on a confocal microscope (Zeiss).
Calcium green mitochondrial Ca\textsuperscript{2+} uptake assays. We measured mitochondrial Ca\textsuperscript{2+} uptake using permeabilized HEK cells as previously described\textsuperscript{33,34}. Briefly, cells were grown in DMEM, with 10% FBS and 1% of Penicillin/Streptomycin. At 80% confluence cells were infected with DN- MUC adeno virus at MOI 10. Cells were harvested after 24h incubation and placed into a 96 well plate. Each well was loaded with 1 million cells in respiratory buffer containing 125.0 mM KCl, 2.0 mM K\textsubscript{2}HPO\textsubscript{4}, 2.0 mM HEPES, 5.0 mM glutamate, 5.0 mM malate, 0.005% saponin, and 5 mM thapsigargin. 5\u00a0\muM Ca\textsuperscript{2+} was injected at 3-min intervals for myocyte experiments.

DN-MUC overexpressing mice. The inter-membrane D\textsubscript{2}MUC\textsubscript{2} homo-monomeric amino acid motif of human MUC was mutated to the dominant-negative (DN) form\textsuperscript{35}, QIMQ, by replacing the nucleotide sequence, 5\textsuperscript{\prime}–GagaGctagcc–3\textsuperscript{\prime} and 5\textsuperscript{\prime}–CggAGcagtc–3\textsuperscript{\prime} (reverse primer containing a C-terminal myc epitope tag and HindIII restriction site (5\textsuperscript{\prime}–AGAGCGAAGCTTACAGGTCTTTCCGCTCAATTGCTGTCATCATTCCACATTTGGCAGAG–3\textsuperscript{\prime}) and reverse primer containing a C-terminal myc epitope tag sequence and HindIII restriction site (5\textsuperscript{\prime}–GAAGCCAGAATTCACCTACAGTTGTCTCGCTTCAACATTGCTGTCATCATTCCACATTTGGCAGAG–3\textsuperscript{\prime}). PCR products were digested and ligated into SalI and HindIII digested pBS-kHGH vector and confirmed by sequencing. Mice embryonic stem cells were injected with the linearized DNA (digested with NotI) in the University of Iowa Transgenic Mouse Core Facility and implanted into pseudo-pregnant females to generate B6XSJL F1 mice. Insertion of the transgene into the mouse genome was confirmed by PCR analysis (not shown) using the forward primer, 5\textsuperscript{\prime}–CggCAGACAGAAATGACAGACAGAT–3\textsuperscript{\prime} and reverse primer, 5\textsuperscript{\prime}–AGAGAAGCAAGAGGCGGATCTA–3\textsuperscript{\prime}, producing a product of 200 bases. Mice were backcrossed to F4 generation or greater into the CD1 background. Transgenic and control mice of either gender were killed at the age of 2–3 months.

Western blots for detecting phospholamban. Heart lysates were prepared from flash-frozen mouse right atria from 6- to 8-week-old female and male mice. Mice were injected with ISO (0.4 mg kg\textsuperscript{-1}) 10 min before harvesting the right atrium and western blotting was performed with a SDS–polyacrylamide gel electrophoresis (SDS–PAGE) electrophoresis system as described\textsuperscript{36}. Briefly, 30 \mug protein samples were size-fractionated on SDS–PAGE, and then transferred to polyvinylidene difluoride membranes. The membranes were probed with anti-phospho-Thr17-PLN (1:5,000), anti-pThr17-PLN (1:5,000) (from Badrilla Ltd., Leeds, UK) at room temperature for 4h. Then membranes were incubated with Alexa-Fluor680-conjugated anti-mouse (Invitrogen Molecular Probes, Carlsbad, CA, USA) and/or IR800Cy5-conjugated anti-rabbit fluorescent secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA, USA) and scanned on an Odyssey infrared scanner (Li-Cor, Lincoln, NE, USA). Integrated densities of protein bands were measured using ImageJ Data Acquisition Software (National Institute of Health, Bethesda, MD, USA). Uncropped scans of all western blot images are available in the SI.

Echocardiography. Transthoracic echocardiography was performed as previously described\textsuperscript{37}. Unanesthetized, sedated mice were used for echocardiography. A 30-MHz linear array transducer was applied to the chest to obtain cardiac images. The transducer was coupled to a Vevo 2100 imager (FUJIFILM VisualSonics, Toronto, Canada). Images of the short and long axis were obtained with a frame rate of ~180–250 hertz. All image analysis was performed offline using Vevo 2100 analysis software (Version 1.5).

ATP measurements. The 6- to 8-week-old littermate mice were killed. Atra were rapidly micro dissected and flash frozen in liquid nitrogen. Atra were homogenized, sonicated and centrifuged at 10,000g. The supernatants were collected and a luciferase assay (Invitrogen, ATP Determination Kit A22066) was used to detect ATP. Samples were measured in triplicate on a Femtotracor FB 12 lumimoremeter (Zylux).

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M.E.A. and Y.W. conceived the project and M.E.A. and Y.W. designed the experiments. Y.W., T.P.R., O.M.K., E.D.L., B.C., Q.W. and A.G.R. performed the experiments, data analysis and interpretations. D.D.H. designed virus constructs for Mt-mericam and DN-MCU. M.A.I. and D.D.H. designed constructs for DN-MCU transgenic mice. L.-S.S. and X.H.T.W. supervised the experiments, data analysis and interpretations, M.E.A. and Y.W. wrote and all the authors edited the manuscript.
Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: Y.W. and M.E.A. are named inventors on a patent application claiming to control excessive heart rates by MCU inhibition.

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Corrigendum: The mitochondrial uniporter controls fight or flight heart rate increases

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In Fig. 4c of this Article, the $y$ axis values were wrongly given as 0, 20, 40, 60 and 80 in the middle and the lower panels. The correct values should read 0, 10, 20, 30 and 40 for the middle and 0, 1, 2, 3 and 4 for the lower panels. In addition, the $y$ axis units of the lower panel were inadvertently changed from F/F0 to Hz/100 while the manuscript was being revised. The correct version of Fig. 4 appears below.

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**Figure 4**

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