Regulation of the Human Cardiac Mitochondrial \( \text{Ca}^{2+} \) Uptake by 2 Different Voltage-Gated \( \text{Ca}^{2+} \) Channels

Guido Michels, MD; Ismail F. Khan, PhD; Jeannette Endres-Becker, PhD; Dennis Rottlaender, MD; Stefan Herzig, MD; Arjang Ruhrparwar, MD; Thorsten Wahlers, MD; Uta C. Hoppe, MD

Background—Impairment of intracellular \( \text{Ca}^{2+} \) homeostasis and mitochondrial function has been implicated in the development of cardiomyopathy. Mitochondrial \( \text{Ca}^{2+} \) uptake is thought to be mediated by the \( \text{Ca}^{2+} \) uniporter (MCU) and a thus far speculative non-MCU pathway. However, the identity and properties of these pathways are a matter of intense debate, and possible functional alterations in diseased states have remained elusive.

Methods and Results—By patch clamping the inner membrane of mitochondria from nonfailing and failing human hearts, we have identified 2 previously unknown \( \text{Ca}^{2+} \)-selective channels, referred to as mCa1 and mCa2. Both channels are voltage dependent but differ significantly in gating parameters. Compared with mCa2 channels, mCa1 channels exhibit a higher single-channel amplitude, shorter openings, a lower open probability, and 3 to 5 subconductance states. Similar to the MCU, mCa1 is inhibited by 200 nmol/L ruthenium 360, whereas mCa2 is insensitive to 200 nmol/L ruthenium 360 and reduced only by very high concentrations (10 \( \mu \text{mol/L} \)). Both mitochondrial \( \text{Ca}^{2+} \) channels are unaffected by blockers of other possibly \( \text{Ca}^{2+} \)-conducting mitochondrial pores but were activated by spermine (1 \( \text{mmol/L} \)). Notably, activity of mCa1 and mCa2 channels is decreased in failing compared with nonfailing heart conditions, making them less effective for \( \text{Ca}^{2+} \) uptake and likely \( \text{Ca}^{2+} \)-induced metabolism.

Conclusions—Thus, we conclude that the human mitochondrial \( \text{Ca}^{2+} \) uptake is mediated by these 2 distinct \( \text{Ca}^{2+} \) channels, which are functionally impaired in heart failure. Current properties reveal that the mCa1 channel underlies the human MCU and that the mCa2 channel is responsible for the ruthenium red–insensitive/low-sensitivity non-MCU–type mitochondrial \( \text{Ca}^{2+} \) uptake. (Circulation. 2009;119:2435-2443.)

Key Words: calcium ■ electrophysiology ■ heart failure ■ mitochondria ■ ion channels

Mitochondrial \( \text{Ca}^{2+} \) transport was discovered in the early 1960s.\(^1\) Mitochondria have been shown to dominate in the clearance of cytosolic \( \text{Ca}^{2+} \)\(^2\) in different cells and are able to accumulate a large amount of \( \text{Ca}^{2+} \).\(^2\) The mitochondrial \( \text{Ca}^{2+} \) uptake from the cytosol controls the rate of energy production,\(^3,4\) modulates the spatial and temporal profile of intracellular \( \text{Ca}^{2+} \) signaling,\(^5–9\) regulates mitochondrial motility and morphology,\(^10\) and may trigger cell death.\(^11\) The \( \text{Ca}^{2+} \) transport into mitochondria is mediated by the \( \text{Ca}^{2+} \) uniporter (MCU), which is blocked by nanomolar concentrations of ruthenium red (RuR) or its analog, ruthenium 360 (Ru360), located in the inner mitochondrial membrane.\(^5,12–15\)

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Mitochondrial \( \text{Ca}^{2+} \) uptake has been evaluated indirectly for several years, ie, by \( \text{Ca}^{2+} \) sensors or measurement of \( \text{Ca}^{2+} \) gradients,\(^5,12,16\) allowing only limited experimental accuracy. Kirichok et al\(^15\) were able to directly record a highly selective mitochondrial \( \text{Ca}^{2+} \) current, which is driven by the negative mitochondrial potential, activated by extramitochondrial \( \text{Ca}^{2+} \) (half-activation constant, \( \approx 20 \text{mmol/L} \); Y\(_{\text{max}} \approx 105 \text{mmol/L} \)) and inhibited by 200 nmol/L RuR or Ru360, thus exhibiting characteristics of the MCU. Because these recordings were performed in mitoplasts (2- to 5-\( \mu \text{m} \) vesicles of inner mitochondrial membrane) from COS-7 cells, results may not readily be transferred to the human setting. Besides the classic MCU, the presence of a second RuR-insensitive mitochondrial \( \text{Ca}^{2+} \) uptake pathway, which is inhibited only by very high RuR concentrations (>1 \( \mu \text{mol/L} \)), has been hypothesized.\(^2,12,17\) Moreover, it is of particular interest whether mitochondrial \( \text{Ca}^{2+} \) channel function is impaired in diseased states associated with profound alterations of intracellular \( \text{Ca}^{2+} \) homeostasis and metabolism such as heart failure.\(^18\)

Therefore, our goal was to directly record and identify channels underlying \( \text{Ca}^{2+} \) influx of human mitochondria.

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From the Department of Internal Medicine III (G.M., I.F.K., J.E.-B., D.R., U.C.H.), Center for Molecular Medicine (S.H., U.C.H.), Institute of Pharmacology (S.H.), and Department of Cardiothoracic Surgery (T.W.), University of Cologne, Cologne, and Department of Cardiac Surgery, University of Heidelberg, Heidelberg (A.R.), Germany.

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.108.835389/DC1. Correspondence to Uta C. Hoppe, MD, Department of Internal Medicine III, University of Cologne, Kerpener-Str 62, 50937 Cologne, Germany. E-mail uta.hoppe@uni-koeln.de
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Second, we intended to evaluate the impact of end-stage heart failure on human mitochondrial Ca\textsuperscript{2+} uptake. Our results demonstrate the existence of 2 different selective Ca\textsuperscript{2+} channels of the human cardiac inner mitochondrial membrane. Furthermore, both mitochondrial Ca\textsuperscript{2+} channels are dysfunctional under heart failing conditions.

**Methods**

**Patients**

Human cardiac mitoplasts were isolated from 15 hearts of patients with end-stage heart failure caused by dilated cardiomyopathy or ischemic cardiomyopathy who were undergoing transplantation. Patient characteristics are given in Table I of the online Data Supplement. Results were compared with mitoplasts prepared from 3 human hearts without heart failure that could not be transplanted for technical reasons (possible systemic infectious disease [n=1], blood group incompatibility [n=2]). Informed consent was obtained before organ transplantation or donation. The study was approved by the local ethics committee.

**Preparation of Myocytes and Mitoplasts**

Single human ventricular myocytes were isolated from nonfailing and failing hearts by enzymatic digestion, as previously described.\textsuperscript{19} Freshly isolated myocytes were used within 1 to 4 hours. Isolated intact mitoplasts were prepared from isolated myocytes by differential centrifugation, as previously reported.\textsuperscript{15,20} For patch-clamp studies, myocytes were labeled with Mitotracker Green 1 \textmu mol/L (Molecular Probes, Inc, Carlsbad, Calif; Figure 1A and Table) to facilitate identification of intact mitoplasts after further subcellular purification.

**Single-Organelle/Cell Reverse-Transcription Polymerase Chain Reaction Analysis**

cDNA synthesis and polymerase chain reaction (PCR) were performed with the OneStep reverse-transcription PCR kit (Qiagen, Hilden, Germany) with some variation from the manufacturer’s directions: 2 isolated mitoplasts or cardiomyocytes were pooled in a reaction buffer. Master mix was prepared as specified in the manual and added to a total reaction volume of 55 \textmu L in 0.5-mL thin-walled PCR tubes. The mixture was incubated at 50°C.

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**Figure 1.** Two distinct voltage-gated mitochondrial Ca\textsuperscript{2+} channels in human cardiac mitoplasts. A, Fluorescence-light microscope photograph of a human cardiac mitoplast (×400) before sealing with the pipette (scale bar=2 \textmu m). Mitoplasts appeared as transparent 2- to 5-\textmu m vesicles with green fluorescence (Mitotracker Green, 1 \textmu mol/L). B, To prove the purity of our mitoplast isolation method, single reverse-transcription PCR experiments were done. The results demonstrate that mitochondrial RNA transcripts (cytochrome C oxidase I [CCO]) could be detected in single (picked) mitoplasts and in lysates of isolated myocytes, whereas GAPDH (a typical genomic RNA transcript) was observed exclusively in cardiomyocytes. C, D, Mitochondrial Ca\textsuperscript{2+} currents at V\textsubscript{m}=-100 mV in a mitoplast-attached configuration demonstrating channels with small openings and high amplitudes (C; I\textsubscript{mCa1}) and currents with wide openings and smaller amplitudes (D; I\textsubscript{mCa2}). I\textsubscript{mCa1} and I\textsubscript{mCa2} could be detected in mitoplasts of nonfailing and failing human hearts, with both channels being less active in mitoplasts from failing myocardium (for data, see text and the Table). E, Occasionally, both channels were present in 1 patch.
Table. Gating Parameters of ImCa1 and ImCa2 From Nonfailing and Failing Hearts

<table>
<thead>
<tr>
<th>Parameter (TP − 100 mv)</th>
<th>mCa1 Nonfailing</th>
<th>n</th>
<th>mCa1 Failing</th>
<th>n</th>
<th>mCa2 Nonfailing</th>
<th>n</th>
<th>mCa2 Failing</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open probability, %</td>
<td>5.43 ± 0.72</td>
<td>3</td>
<td>3.11 ± 0.34*</td>
<td>6</td>
<td>23.8 ± 4.17††</td>
<td>3</td>
<td>6.02 ± 0.91*</td>
<td>5</td>
</tr>
<tr>
<td>Availability, %</td>
<td>50.0 ± 7.71</td>
<td>3</td>
<td>32.8 ± 2.95*</td>
<td>6</td>
<td>48.2 ± 3.18††</td>
<td>3</td>
<td>28.4 ± 5.42*</td>
<td>5</td>
</tr>
<tr>
<td>Mean open time, ms</td>
<td>0.31 ± 0.05</td>
<td>3</td>
<td>0.27 ± 0.01</td>
<td>6</td>
<td>5.08 ± 0.70††</td>
<td>3</td>
<td>2.73 ± 0.51††</td>
<td>5</td>
</tr>
<tr>
<td>Mean closed time, ms</td>
<td>3.62 ± 0.64</td>
<td>3</td>
<td>5.22 ± 0.32</td>
<td>6</td>
<td>7.10 ± 1.03††</td>
<td>3</td>
<td>8.21 ± 0.92††</td>
<td>5</td>
</tr>
<tr>
<td>Mean first latency, ms</td>
<td>39.7 ± 3.48</td>
<td>3</td>
<td>56.5 ± 4.16*</td>
<td>6</td>
<td>34.8 ± 5.84††</td>
<td>3</td>
<td>67.0 ± 3.30††</td>
<td>5</td>
</tr>
<tr>
<td>Amplitude, pA</td>
<td>−1.43 ± 0.07</td>
<td>3</td>
<td>−1.06 ± 0.08*</td>
<td>6</td>
<td>−0.63 ± 0.05††</td>
<td>3</td>
<td>−0.61 ± 0.06††</td>
<td>5</td>
</tr>
<tr>
<td>I peak, fA</td>
<td>−49 ± 13</td>
<td>3</td>
<td>−12 ± 3*</td>
<td>6</td>
<td>−153 ± 33††</td>
<td>3</td>
<td>−12 ± 4††</td>
<td>5</td>
</tr>
<tr>
<td>Conductance, pS</td>
<td>13.7 ± 0.78</td>
<td>3</td>
<td>10.9 ± 0.33*</td>
<td>6</td>
<td>7.67 ± 0.80††</td>
<td>3</td>
<td>6.56 ± 0.74††</td>
<td>5</td>
</tr>
<tr>
<td>Ca2+ permeability, 10−14 cm2/s</td>
<td>5.60 ± 0.13</td>
<td>3</td>
<td>4.10 ± 0.22*</td>
<td>6</td>
<td>0.44 ± 0.02††</td>
<td>3</td>
<td>0.36 ± 0.02††</td>
<td>5</td>
</tr>
<tr>
<td>Ca2+ flux rate, 10−6 s−1</td>
<td>3.50 ± 0.16</td>
<td>3</td>
<td>3.50 ± 0.12</td>
<td>6</td>
<td>2.20 ± 0.18††</td>
<td>3</td>
<td>2.10 ± 0.14††</td>
<td>5</td>
</tr>
</tbody>
</table>

*P<0.05 versus respective nonfailing; †P<0.05 versus mCa1 nonfailing; ‡P<0.05 versues mCa1 failing.

Holding potential, −40 mV; test potential (TP), −100 mV. Ipeak was measured from ensemble averages (see Methods).

Sublevel Analysis of Single-Channel Data

Pmax (maximum of simultaneously open channels) is an estimator for n, the exact number of channels in a patch with k simultaneous detected openings (stacked current levels). Pmax is reliable only for patches where k ≤ 5, as included here for analysis (CDE-REVLEV/LX program, version 1.3). The Pmax probability, Prob (channel n=k), is then given by the following: Prob (n=k)=Pmax (k, Pm, M)=1−(1−Pm)M, where k is the number of simultaneous openings or sublevels, n is the total number of channels or sublevels, Pm is the single-channel open probability calculated over the entire recording time, and M is the total number of sweeps.

Results

Patient Characteristics

To directly analyze the Ca2+ uptake in human mitochondria and to assess the impact of heart failure on mitochondrial Ca2+ channel function, we patch clamped single cardiac mitoplasts (Figure 1A and 1B) of 18 human hearts (supplementary Table I). To omit any contamination by mitochondria of nonmyocytes, we performed a 2-step isolation process, ie, isolation of human cardiomyocytes, which they then were used for mitoplast generation.

Human Mitochondrial Ca2+ Uptake Is Regulated by 2 Different Mitochondrial Ca2+ Channels

Consistent with previous single Ca2+ channel recordings of mitoplasts from COS-7 cells,19 we also chose a [Ca2+]S Psys of 105 mmol/L. In the mitoplast-attached configuration, voltage steps to −100 mV elicited 2 distinct types of voltage-dependent Ca2+ currents (Figure 1C and 1D) that we refer to as lCa1 and lCa2 (for mitochondrial Ca2+ current type 1 and 2). Occasionally, both Ca2+ currents could be recorded in same patches (Figure 1E), indicating that mCa1 and mCa2 opening, and lCa2 (the peak ensemble average current, either fitted by an adapted Hodgkin-Huxley model if possible [continuously differentiable] or computed by the local minimum method, as given previously21) were calculated only from single-channel patches. To determine single-channel amplitudes most accurately, amplitudes were obtained not only by direct measurements of visually fully resolved openings and as the maximum of gaussian fits on amplitude histograms but also by the use of a peak integral differential detector (supplemental Methods). Despite this evaluation, we may not entirely exclude that the full conductance of channels with spike-like gating kinetics might have been underestimated.

Single-Channel Recordings

All experiments were performed in the mitoplast-attached configuration of the patch-clamp technique (test pulse, 150-ms duration at 1.67 Hz; sampling frequency [fS], 10 kHz; cutoff frequency [fc], 2 kHz), as previously described.20 Only experiments with a high seal resistance between 40 and 80 GΩ were included. The bath solution contained 160 mmol/L KCl, 10 mmol/L HEPES, 1 mmol/L EDTA, and 1 mmol/L EGTA (pH 7.2 with KOH). Pipettes were filled with a solution composed of 105 mmol/L CaCl2 (if not otherwise indicated), 10 mmol/L HEPES [pH 7.2 with Ca(OH)2]. Specific drugs were added to solutions to block the mitochondrial permeability transition pore (cyclosporin A, Sigma Aldrich, St Louis, Mo; 10 μmol/L), the mitochondrial ryanodine receptor (dantrolene, Sigma Aldrich; 10 μmol/L), the IP3R (xestospongin C, Sigma Aldrich; 10 μmol/L), the mitochondrial Na+/Ca2+ exchanger (CGP-37157, Calbiochem, San Diego, Calif; 10 μmol/L), and the mCa1/2 (Ru360, Calbiochem; 200 nmol/L or 10 μmol/L) as indicated. Sperrmine (Sigma Aldrich; 1 mmol/L) was added to activate mCa1/2, and Sc3+-BayK 8644 (Sigma Aldrich; 10 μmol/L) was added to rule out that mitochondrial Ca2+ channels are 1,4-dihydropyridine sensitive. Currents were recorded and digitized with an Axopatch 200B amplifier and Digidata 1200 interface (Axon Instruments, Foster City, Calif).

Single-Channel Data Analysis

Single-channel analysis was done with custom software (CDE-REVLEV/LX program, version 1.3), as previously reported21 and further extended for this work (supplemental Methods). Linear leak and capacity currents were digitally subtracted using the average currents of nonactive sweeps. For detailed gating analysis, idealized currents were analyzed in 150-ms steps. The open probability (defined as the occupancy of the open state during active sweeps), the availability (fraction of sweeps containing at least 1 channel opening), and lCa2 (the peak ensemble average current, either fitted by an adapted Hodgkin-Huxley model if possible [continuously differentiable] or computed by the local minimum method, as given previously21) were calculated only from single-channel patches. To determine single-channel amplitudes most accurately, amplitudes were obtained not only by direct measurements of visually fully resolved openings and as the maximum of gaussian fits on amplitude histograms but also by the use of a peak integral differential detector (supplemental Methods). Despite this evaluation, we may not entirely exclude that the full conductance of channels with spike-like gating kinetics might have been underestimated.
channels are not separately present and thus characteristic for different mitochondrial subpopulations. These 2 Ca\(^{2+}\) currents differed in several single-channel parameters (the Table and Figure 1C and 1D). Compared with mCa1 channels, mCa2 channels had a smaller single-channel amplitude, longer openings (mean open times), and a higher open probability. Moreover, mCa1 and mCa2 channels exhibited a distinct bursting behavior (mCa2>mCa1; supplemental Table II). Activity of both mitochondrial Ca\(^{2+}\) channels increased at more negative voltages (higher open probability and availability, shorter mean first latency) (Figure 2C), making these channels effective for Ca\(^{2+}\) respiration at more negative potentials (higher open probability). Moreover, mCa1 and mCa2 channels exhibited a longer opening (mean open times), and a higher open probability. mCa2 channels had a smaller single-channel amplitude, whereas the Ca\(^{2+}\) permeability of both Ca\(^{2+}\) channels differed in several single-channel parameters (the Table). To exclude the possibility that our biophysical analysis of mCa1 versus mCa2 channels might be biased by a selection artifact, we plotted two of the proposed distinctive biophysical features, ie, single-channel conductance and mean closed time (at V\(_{\text{m}}\) = −100 mV), against each other (Figure 2B). No single case of overlap based on these 2 independent parameters further supports the distinct identity of the 2 Ca\(^{2+}\) currents.

To show that the amplitudes of mCa1 taken as peaks are averages of multiple points (≥1 sample point), we performed additional recordings at higher bandwidth (sampling frequency, 25 kHz; cutoff frequency, 5 kHz) (Figure 3A). However, a wider bandwidth of 5 kHz did not provide any improvement in single-channel detection in unfiltered data or its post--digital-filtered correlate (8-pole Bessel filter; f\(_{c}\) = 2 kHz) compared with 2-kHz analog-filtered data. Rather, a higher filter frequency of 5 kHz created a higher root mean square background noise, a reduced signal-to-noise ratio, a higher number of spurious and false events, and digital postfiltered sweeps, which were rarely distinguishable from their 2-kHz analog-filtered counterparts.

Gating and Permeability of Both Mitochondrial Ca\(^{2+}\) Channels Are Suppressed Under Heart Failure Conditions

ImCa1 and ImCa2 could be detected in mitoplasts from both nonfailing and failing human hearts (Figure 1C and 1D). Notably, under failing heart conditions, the single-channel activity and gating of mCa1 and mCa2 were reduced, particularly exhibiting a lower open probability, a longer mean closed time, and a prolonged mean first latency, compared with nonfailing mitochondrial Ca\(^{2+}\) channels (Figures 1C, 2A, and 2C; supplemental Figure I; and the Table). Moreover, we obtained a decrease in Ca\(^{2+}\) permeation strength and Ca\(^{2+}\) permeability of both Ca\(^{2+}\) channels under failing conditions, whereas the Ca\(^{2+}\) flux rate was unchanged (the Table).

Kinetics of Human Mitochondrial Ca\(^{2+}\) Uptake

Because the kinetics of mitochondrial Ca\(^{2+}\) uptake are currently a matter of intense debate,\(^{2,6,17}\) we analyzed the cumulative first latencies (time of first activation [\(\tau_1\)]) and steady-state activation [\(\tau_2\)]) and found that under nonfailing conditions the Ca\(^{2+}\) uptake via mCa1 and mCa2 channels is rapid, whereas in mitoplasts from failing hearts, a slower first activation and steady-state activation were evident (Figure 2D), consistent with reports from >30 years ago that mitochondrial Ca\(^{2+}\) uptake in failing hearts is decreased compared with control conditions.\(^{24,25}\)

![Figure 2. Biophysical properties of mitochondrial Ca\(^{2+}\) channels in human cardiac mitoplasts. A, Single-channel amplitude as a function of the test potential. Slope conductances as depicted here were calculated from pooled data (n = 3 to 6). See text for average values determined by linear regression in each individual experiment. B, Comparison between ImCa1 and ImCa2 using 2 independent biophysical criteria: single-channel conductance (abscessia) and mean closed time (ordinate). There is no overlap between the values covered by each criterion, and there is no case of ambiguity, as indicated by the 2 empty quadrants (open symbols, nonfailing; filled symbols, failing). C, Gating behavior of both mitochondrial Ca\(^{2+}\) channels at different test potentials (holding potential, −40 mV) of nonfailing (NF) and failing (F) human heart mitoplasts. Gating parameters: open probability (Po [%]), availability (Avail [%]), mean open time (MOT [ms]), mean closed time (MCT [ms]), and mean first latency (MFL [ms]) (n = 3 to 6). D, Cumulative first latency function at V\(_{\text{m}}\) = −100 mV. Both channels from nonfailing hearts open significantly earlier (ImCa1: \(\tau_1\) = 28.9 ± 0.01 ms [24 ± 8%], \(\tau_2\) = 181 ± 0.03 ms [76 ± 5%]; ImCa2: \(\tau_1\) = 5.48 ± 0.01 ms [38 ± 2%], \(\tau_2\) = 156 ± 0.02 ms [82 ± 1%]) compared with failing hearts (ImCa1: \(\tau_1\) = 44.7 ± 0.03 ms [2 ± 0.1%], \(\tau_2\) = 314 ± 0.02 ms [98 ± 0.8%]; ImCa2: \(\tau_1\) = 9.36 ± 0.04 ms [3 ± 0.6%], \(\tau_2\) = 419 ± 0.01 ms [97 ± 0.2%]) (n = 3 to 6).
open levels of mCa1 are clearly from 1 single channel and are not caused by multiple openings of >1 channel (supplemental Table III). Notably, I_mCa from nonfailing hearts exhibited 3 sublevels, which were increased to 5 sublevels under failing heart conditions, suggesting impaired opening of mCa1 channels in heart failure.

Ca\(^{2+}\) Selectivity of Both Mitochondrial Ca\(^{2+}\) Channels

The main intracellular monovalent cation, ie, potassium, does not seem to contribute to I_{mCa} or I_{mCa2} in the presence of Ca\(^{2+}\).

The conductances of mCa1 and mCa2 increase with increasing [Ca\(^{2+}\)]_pipette (g_{mCa1}: 7.1±0.43 pS [20 mmol/L, n=3], 9.8±0.89 pS [52.5 mmol/L, n=4], 10.9±0.33 pS [105 mmol/L, n=6]; g_{mCa2}: 3.9±0.52 pS [20 mmol/L, n=3], 6.08±0.42 pS [52.5 mmol/L, n=3], 6.56±0.74 pS [105 mmol/L, n=5]), revealing a half-saturating concentration, K_{m}, of 15.1±0.03 mmol/L Ca\(^{2+}\) (n=3 to 6) and 19.6±0.20 mmol/L Ca\(^{2+}\) (n=3 to 5; P<0.05) for mCa1 and mCa2, respectively (Figure 3B and 3C). The single-channel conductance of both channels in the presence of K\(^+\) at the cytoplasmic surface (pipette solution: 105 mmol/L K\(^+\), 52.5 mmol/L Ca\(^{2+}\)) was not significantly changed compared with Ca\(^{2+}\) (52.5 mmol/L) alone [failing: g_{mCa1} (K\(^+\)-Ca\(^{2+}\)), 10.3±1.15 pS ([n=3; P=NS versus g_{mCa1} (Ca\(^{2+}\))]; g_{mCa2} (K\(^+\)-Ca\(^{2+}\)), 5.80±0.62 pS ([n=3; P=NS versus g_{mCa2} (Ca\(^{2+}\))]) (Figure 3B and 3D). Furthermore, the reversal potential was unaffected by extramitochondrial K\(^+\) [V_{rev} of I_{mCa1} (K\(^+\)-Ca\(^{2+}\)), 21.2±1.24 mV (n=3) versus V_{rev} of I_{mCa1} (52.5 mmol/L Ca\(^{2+}\)), 21.0±0.88 mV (n=4; P=NS); V_{rev} of I_{mCa2} (K\(^+\)-Ca\(^{2+}\)), 10.6±1.09 mV (n=3) versus V_{rev} of I_{mCa2} (52.5 mmol/L Ca\(^{2+}\)), 9.20±0.50 mV (n=3; P=NS)] (Figure 3B and 3D). These data support that mCa1 and mCa2 channels are less permeant or nonpermeant for K\(^+\) in the presence of Ca\(^{2+}\), thus preventing mitochondrial depolarization by these abundant cations.\(^{15}\)

To rule out that the mitochondrial Ca\(^{2+}\) channels mCa1 and mCa2 conduct anions, we performed experiments with 105 mmol/L Ca\(^{2+}\)-glutamate in the pipette solution (Figures 3A and 3D). Under these conditions, we observed the same channel behaviors and single-channel conductances as in the presence of Cl\(^-\), ie, with 105 mmol/L CaCl\(_2\) in the pipette solution [failing: g_{mCa1} (Ca\(^{2+}\)-glutamate), 10.1±0.29 pS (n=4; P=NS versus g_{mCa1} (Ca\(^{2+}\))]; g_{mCa2} (Ca\(^{2+}\)-glutamate), 5.92±0.20 pS (n=4; P<0.05 versus g_{mCa2} (Ca\(^{2+}\))); P=NS versus g_{mCa2} (Ca\(^{2+}\))], supporting that mCa1 and mCa2 were not conducting Cl\(^-\).

Pharmacological Characteristics of Both Mitochondrial Ca\(^{2+}\) Channels

Reliably evaluating the effect of modulators channel stability over time is important. Under control conditions, mCa1 and mCa2 were stable over a recording time of 20 minutes (no rundown; Figure 5A). Similar to the mitochondrial Ca\(^{2+}\) uptake via the MCU,\(^{2,15,17}\) I_{mCa} was inhibited by 200 mmol/L Ru360 (Figure 5B and 5C). I_{mCa2} was unaffected by 200 mmol/L Ru360 but was significantly suppressed by 10 μmol/L Ru360 (Figure 5B and 5C), consistent with reports suggesting an RuR-insensitive/low-sensitivity mitochondrial Ca\(^{2+}\) uptake in addition to the classic RuR-sensitive MCU.\(^{2,12,16}\) These data were derived from mitoplasts under...
nonfailing heart conditions. Pharmacological experiments of mitoplasts from failing hearts showed a more pronounced Ru360 inhibition (Figure 5D and 5E).

To further support the identity of mCa1 and mCa2, we performed additional agonist experiments. Spermine (1 mmol/L), an activator of the MCU,26 significantly increased both channel activities that were blocked—after washout—by 10 μmol/L Ru360 (Figure 5D and 5E). The pharmacological effects were stronger for mCa1 than for mCa2, supporting that mCa1 is the RuR-sensitive MCU.

Although the remaining Ca2+ uptake in the presence of nanomolar concentrations of Ru360 might be due to alternative Ca2+ pathways via the mitochondrial Na+-Ca2+ exchanger12,27 or mitochondrial ryanodine receptor,28 addition of CGP-37157 (10 μmol/L), cyclosporin A (10 μmol/L), dantrolene (10 μmol/L), 5(-)-BayK 8644 (10 μmol/L), or xestospongic acid (10 μmol/L) to the bath or pipette solution did not affect ImCa1 or ImCa2 (supplemental Table IV), indicating that these 2 Ca2+ currents were not mediated by the mitochondrial Na+-Ca2+ exchanger,29 mitochondrial permeability transition pore,30 mitochondrial ryanodine receptor,28 mitochondrial dihydropyridine site,31 or an IP3 receptor,32 respectively.

**Discussion**

The present study demonstrates that 2 distinct ion channels underlie human mitochondrial Ca2+ sequestration. Both channels are voltage gated, are Ca2+ selective in the presence of the predominant intracellular monovalent cation K+ and the anion Cl−, and exhibit increased activity at more negative potentials, making them particularly sufficient for Ca2+ uptake driven by the large electrochemical gradient for Ca2+ across the inner membrane of energized mitochondria. mCa1 and mCa2 channels can clearly be distinguished by their unique single-channel characteristics and different sensitivity to Ru360, explaining previous reports of mitochondrial Ca2+ uptake via the MCU and a non-MCU pathway.2,12,17

Human mCa1 channels share some properties with MiCa channels recorded in COS-7 cells, ie, a similar range of single-channel conductance, amplitude, and Ca2+ flux, an increased open probability at more negative voltages, subconductance states, and sensitivity to nanomolar Ru360 concentrations, but differ markedly in gating parameters like open and closed times, suggesting species- and/or tissue-specific diversity of the MCU.15 Although suppression of the so-called non-MCU–type, RuR-insensitive Ca2+ uptake in
mammalian mitochondria by high RuR concentrations led to
the assumption that it might be mediated by a MCU with
reduced RuR sensitivity,2,12,17 distinct single-channel proper-
ties of ImCa1 and ImCa2, even when recorded simultaneo-
sily in 1 mitoplast, indicate that these 2 channels are composed of
different protein complexes. Previously, a higher Ru360
sensitivity has been obtained for mitochondrial Ca2+
uptake
linked to the MCU with complete suppression by a concen-
tration of 200 nmol/L.2,12–15 However, we do not consider
these and our results contradictory because the difference in
inhibition may be due, at least partly, to different experimen-
tal conditions, given that for other channels and compounds,
a lower sensitivity in cell-attached versus whole-cell config-
uration also has been reported.33,34 Moreover, activation of
both mCa1 and mCa2 by spermine further supports their Ca2+
channel identity of the MCU and non-MCU type.26

Although mitochondrial dysfunction in disease and aging
has been related mainly to direct alterations of the respira-
tory chain or ΔΨ,35,36 here we demonstrate reduced function of
mitochondrial Ca2+-channels and thus of an indirect modula-
tor of energy and reactive oxygen species production in a
disease known to be associated with profound changes in
intracellular Ca2+ homeostasis and metabolism.18 Alterations
of the mitochondrial matrix composition (ie, Ca2+ buffering/
alkalization, matrix-free [Ca2+]). H+ translocation, protein
expression) might also have contributed to the observed
differences in mCa1/2 channel properties of mitochondria
from failing and nonfailing hearts in the present study.
Moreover, a depolarization of the potential across the inner
mitochondrial membrane, which has been reported for car-
diomyopathic hamsters, might have decreased the driving
force and thus Ca2+ channel activity under failing heart
conditions.36 However, in addition to differences in the basal
biophysical channel behavior, we obtained marked differ-
ences in the pharmacological behavior under nonfailing
(Figure 5B and 5C) and failing (Figure 5D and 5E) condi-
tions, supporting the hypothesis of (additional) changes in
channel structure, subunit composition, or binding sites in
diseased state. In this respect, a possible contribution of
altered expression levels of uncoupling proteins 2/3, which
recently have been implicated in the function of the MCU,
have to be determined.12,37,38

Maintaining a high ATP supply is critically important for
maintaining cardiac performance. The daily turnover of ATP
in the heart is very many times that of the myocardial ATP
pool. Thus, even subtle variations in the efficiency of energy
generation and use may have a cumulative impact on cellular
energy levels. Numerous studies have identified decreased
cardiac energy levels and flux as consistent features of heart
failure.39,40 Analysis of human biopsy specimens demon-
strated that ATP is 25% to 30% lower in failing human hearts
compared with controls.41,42 Although the myocardial tissue
is well oxygenized, it remains to be determined whether the chronic, progressive loss of ATP in heart failure occurs because de novo synthesis of ATP is slowed or fails because ATP degradation is accelerated. Our observations of suppressed mitochondrial Ca\(^{2+}\) channel activity support the notion that this mismatch might be due, at least partly, to a reduced mitochondrial Ca\(^{2+}\) uptake and thus Ca\(^{2+}\)-induced ATP generation.\(^3\)\(^,\)\(^4\) Only a few reports are contrary to this dogma that mitochondrial Ca\(^{2+}\) accumulation accelerates mitochondrial metabolism.\(^4\)\(^,\)\(^4\)\(^4\)\(^,\)\(^4\)\(^5\) In these experiments, conditions are present in which the depolarizing effect of Ca\(^{2+}\) on the inner mitochondrial membrane exceeds its stimulatory effect on the respiratory chain and dehydrogenases and consequently leads to a Ca\(^{2+}\)-induced decrease in NADH levels. In such a scenario, reduced mitochondrial Ca\(^{2+}\) channel activity would prevent further mitochondrial Ca\(^{2+}\) overload and thus decline in ATP synthesis.

Furthermore, mitochondrial Ca\(^{2+}\) uptake exhibits dual contribution in the regulation and control of reactive oxygen species production, which has been implicated in triggering mitochondrial DNA mutations and cell death.\(^4\)\(^6\) Mitochondrial Ca\(^{2+}\) sequestration has been reported to facilitate the generation of reactive oxygen species within mitochondria,\(^4\)\(^7\) whereas dissipating ΔΨ on excessive Ca\(^{2+}\) accumulation is expected to counteract generation of ·O\(_2\) during oxidative phosphorylation.\(^4\)\(^8\)\(^,\)\(^4\)\(^9\) Therefore, it remains unclear whether suppressed activity of mCa1 and mCa2 in human heart failure should be considered adaptive, maladaptive, or a cause of progressive decline in pump function.

**Conclusions**

This study demonstrates for the first time that human cardiac mitochondrial Ca\(^{2+}\) uptake is regulated by 2 different selective mitochondrial Ca\(^{2+}\) channels: mCa1, the RuR-sensitive Ca\(^{2+}\) uniporter, and mCa2, an RuR-insensitive/low-sensitivity non-MCU–type mitochondrial Ca\(^{2+}\) channel. Furthermore, we found that both mitochondrial Ca\(^{2+}\) currents are less active under failing heart conditions than in mitochondria from non-failing hearts.

On the basis of our experiments, we conclude that the human MCU and the RuR-insensitive Ca\(^{2+}\) uptake pathway are the Ca\(^{2+}\)-selective ion channels mCa1 and mCa2, respectively, which are functionally impaired in the diseased heart state.

**Acknowledgments**

We thank Nadine Henn and Iris Berg for technical assistance.

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**Disclosures**

None.

**References**

Impairment of intracellular Ca\(^{2+}\) homeostasis and mitochondrial function has been implicated in the development of cardiomyopathy. Numerous studies have identified decreased cardiac energy levels and flux as consistent features of heart failure. The mitochondrial Ca\(^{2+}\) uptake from the cytosol controls, among other things, the rate of energy production. Mitochondrial Ca\(^{2+}\) sequestration is thought to be mediated by the Ca\(^{2+}\) uniporter (MCU) and a thus far speculative non-MCU pathway. However, the identity and properties of these pathways are a matter of intense debate, and possible functional alterations in diseased states have remained elusive. By patch clamping the inner membrane of mitochondria from nonfailing and failing human hearts, we have demonstrated that human cardiac mitochondrial Ca\(^{2+}\) uptake is regulated by 2 different selective Ca\(^{2+}\) channels that we refer to as mCa1 and mCa2. Both channels are voltage gated and exhibit increased activity at more negative potentials, making them particularly sufficient for Ca\(^{2+}\) uptake driven by the large electrochemical gradient for Ca\(^{2+}\) across the inner membrane of energized mitochondria. The distinct biophysical and pharmacological properties of these 2 Ca\(^{2+}\) channels support the notion that they underlie the human MCU and the non-MCU Ca\(^{2+}\) uptake pathway, respectively. Notably, activity of mCa1 and mCa2 channels is decreased in failing compared with nonfailing heart conditions, making them less effective for Ca\(^{2+}\) uptake and likely Ca\(^{2+}\)-induced metabolism, which may explain, at least partly, reduced myocardial ATP levels in advanced heart failure.
Regulation of the Human Cardiac Mitochondrial Ca\textsuperscript{2+} Uptake by 2 Different Voltage-Gated Ca\textsuperscript{2+} Channels

Guido Michels, Ismail F. Khan, Jeannette Endres-Becker, Dennis Rottlaender, Stefan Herzig, Arjang Ruhparwar, Thorsten Wahlers and Uta C. Hoppe

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SUPPLEMENTAL MATERIAL

Supplemental Methods

**Single-channel data analysis.** Single-channel analysis was done using custom software (CDE-REVL-LEVL/X program, Version 1.3) as previously reported\(^1\) and further extended for this work.

To determine open state (level) amplitudes our single-channel detection includes (i) time-course fitting combined with additional (ii) PID algorithms (Peak-Integral-Differential detector):

- time course fitting: a rectangular step input function to fit assumed square events supported by polynomial-fitting function for the discrimination between RMS background noise and channel events,
- peak detector: find peaks from the baseline level given a definite signal-to-noise ratio,
- differential (slope) detector: find transitions using the first, second and third derivative of the recording function \(I(t)\) to explore sublevels,
- integral (area) detector: validate our transitions from differential analysis using the time integral of \(I(t)\).

All these steps were integrated in our analysing program and all our data were put into this multifunctional program suite (CDE, **Channel Data Evaluation, 2001-2008**).

Time constants of open time \((\tau_{\text{open}})\) and closed time histograms \((\tau_{\text{closed}})\) were obtained by simplex maximum likelihood estimation on all-level open and closed time distributions. The number of kinetic states within the model was determined by the “best fit” method and judged by the LLR ratios\(^1,2\).

Cumulative first latencies (Figure 2D) were transformed to probabilities and subsequently fitted by biexponential functions given by

\[
\frac{1}{M_0} N(t) = \frac{1}{M_0} \left[ P_1 \left( 1 - e^{-\frac{t}{\tau_1}} \right) + P_2 \left( 1 - e^{-\frac{t}{\tau_2}} \right) \right].
\]

Fitting was done using Levenberg-Marquardt least-square-root method built in CDE-REVL-LEVL/X and pSTAT programs. \(N\): number of cumulated active traces divided through the
total number of sweeps at single times \( t \); \( P_1, P_2 \): proportions of exponential components; \( \tau_1 \): fast component of latency (time of first activation), \( \tau_2 \): slow component of latency (time of saturation/steady state activation); \( M_0 \): total number of sweeps.

**Single-channel conductance.** The single-channel conductance was calculated individually by linear regression.

**Permeabilities.** Ion permeabilities were determined by fitting I-V curves using the Goldman-Hodgkin-Katz current-flux equation (Hille, 2001), modified by introducing an additional surface potential shift to obtain better approximations is some cases (adapted from Smith et al., 1993):

\[
i(V) = P_s \cdot \left( \frac{z_i F}{RT} \right) \cdot \left( \frac{V - V_s}{V_v} \right) \cdot e^{-\left( \frac{z_i F}{RT} V \right)} \cdot \frac{S_0 - S_i \cdot e^{-\left( \frac{z_i F}{RT} V_s \right)}}{1 - e^{-\left( \frac{z_i F}{RT} V \right)}},
\]

Abbreviations: \( P_s \): permeability (cm\(^3\) s\(^{-1}\)); \( V_s \): surface potential shift (mV), \( V \): test potential (mV), \( S_0 \): external ion concentration (mM), \( S_i \): internal ion concentration (mM), \( z_i \): ion valence, \( R \): gas constant, \( F \): Faraday constant. \( V_{rev} \) (reversal potential, mV) was detected by zero-current potential when current < RMS amplitude resolution (0.12 pA).

**Ion flux rate.** The \( \text{Ca}^{2+} \) flux rate was calculated by \( \Phi_{max} = i l (z \cdot Q_e) \), with \( i \), amplitude; \( z \), ion valence; \( Q_e \), elementary charge. Pooled data are presented as mean±s.e.m. An unpaired two-tailed \( t \) test was used for statistical examinations. Probability values of \( p<0.05 \) were deemed significant.
### Supplemental Table 1

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Sex</th>
<th>Age (years)</th>
<th>HTX-diagnosis</th>
<th>Medication</th>
<th>CI (l/min/m$^2$)</th>
<th>EF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R.P.</td>
<td>f</td>
<td>51</td>
<td>NF (SAH)</td>
<td>-</td>
<td>-</td>
<td>85</td>
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<tr>
<td>I.K.</td>
<td>m</td>
<td>55</td>
<td>NF (SAH)</td>
<td>-</td>
<td>-</td>
<td>90</td>
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<tr>
<td>H.S.</td>
<td>m</td>
<td>39</td>
<td>NF (ICH)</td>
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<td>-</td>
<td>88</td>
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<tr>
<td>S.B.</td>
<td>m</td>
<td>56</td>
<td>ICM</td>
<td>A, B, C, D, E</td>
<td>1.7</td>
<td>19</td>
</tr>
<tr>
<td>I.M.</td>
<td>f</td>
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<td>DCM</td>
<td>A, B, C, F</td>
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<td>15</td>
</tr>
<tr>
<td>K.M.</td>
<td>m</td>
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<td>DCM</td>
<td>A, B, C, F</td>
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<td>10</td>
</tr>
<tr>
<td>D.M.</td>
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<td>67</td>
<td>ICM</td>
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<td>15</td>
</tr>
<tr>
<td>G.K.</td>
<td>m</td>
<td>57</td>
<td>ICM</td>
<td>A, B, C, E, F</td>
<td>1.2</td>
<td>18</td>
</tr>
<tr>
<td>P.S.</td>
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<td>27</td>
<td>DCM</td>
<td>A, B, C, E, F</td>
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<td>16</td>
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<tr>
<td>S.O.</td>
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<td>DCM</td>
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<td>16</td>
</tr>
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<td>I.H.</td>
<td>m</td>
<td>58</td>
<td>DCM</td>
<td>A, B, C, E, F</td>
<td>1.1</td>
<td>20</td>
</tr>
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<td>M.M.</td>
<td>m</td>
<td>44</td>
<td>DCM</td>
<td>A, B, C, F</td>
<td>1.1</td>
<td>18</td>
</tr>
<tr>
<td>M.H.</td>
<td>f</td>
<td>38</td>
<td>DCM</td>
<td>A, B, C, D, E</td>
<td>1.2</td>
<td>10</td>
</tr>
<tr>
<td>E.L.</td>
<td>f</td>
<td>61</td>
<td>DCM</td>
<td>A, B, C, D</td>
<td>1.8</td>
<td>16</td>
</tr>
<tr>
<td>W.F.</td>
<td>m</td>
<td>56</td>
<td>DCM</td>
<td>A, B, C, D, E, F</td>
<td>1.6</td>
<td>14</td>
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<tr>
<td>K.J.</td>
<td>m</td>
<td>45</td>
<td>DCM</td>
<td>A, B, C, E, F</td>
<td>1.3</td>
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<td>H.P.</td>
<td>m</td>
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<td>DCM</td>
<td>A, B, C, E, F</td>
<td>1.1</td>
<td>15</td>
</tr>
<tr>
<td>M.S.</td>
<td>m</td>
<td>49</td>
<td>DCM</td>
<td>A, B, C, F</td>
<td>1.4</td>
<td>17</td>
</tr>
</tbody>
</table>

**Supplemental Table 1.** Patients’ characteristics. Abbreviations: m, male; f, female; CI, cardiac index; DCM, dilated cardiomyopathy; EF, ejection fraction; ICM, ischemic cardiomyopathy; NF, non-failing (SAH, traumatic subarachnoid hemorrhage; ICH, traumatic intracranial hemorrhage); medications: A, ACE inhibitor/AT1 receptor blocker; B, ß-blocker; C, diuretics; D, digitalis glycoside; E, Amiodarone; F, other.
**Supplemental Table 2**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>mCa1 non-failing</th>
<th>n</th>
<th>mCa1 failing</th>
<th>n</th>
<th>mCa2 non-failing</th>
<th>n</th>
<th>mCa2 failing</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean burst open duration (ms)</td>
<td>8.90 ± 0.82</td>
<td>3</td>
<td>4.57 ± 1.10</td>
<td>6</td>
<td>41.0 ± 2.41*‡</td>
<td>3</td>
<td>11.1 ± 1.56‡</td>
<td>5</td>
</tr>
<tr>
<td>Mean burst closed duration (ms)</td>
<td>52.6 ± 4.36</td>
<td>3</td>
<td>38.7 ± 3.71</td>
<td>6</td>
<td>43.8 ± 3.60</td>
<td>3</td>
<td>35.9 ± 2.69#</td>
<td>5</td>
</tr>
<tr>
<td>Intraburst P_o (%)</td>
<td>10.2 ± 0.42</td>
<td>3</td>
<td>10.6 ± 0.70</td>
<td>6</td>
<td>48.3 ± 2.48‡</td>
<td>3</td>
<td>23.5 ± 1.90*‡</td>
<td>5</td>
</tr>
<tr>
<td>Interburst P_o (%)</td>
<td>0.20 ± 0.01</td>
<td>3</td>
<td>0.55 ± 0.07</td>
<td>6</td>
<td>3.80 ± 0.70#†</td>
<td>3</td>
<td>4.70 ± 0.94#‡</td>
<td>5</td>
</tr>
<tr>
<td>Mean openings per burst</td>
<td>30.0 ± 3.21</td>
<td>3</td>
<td>16.7 ± 2.60*</td>
<td>6</td>
<td>19.0 ± 2.07‡</td>
<td>3</td>
<td>5.70 ± 0.89#</td>
<td>5</td>
</tr>
<tr>
<td>Mean closures per burst</td>
<td>21.8 ± 2.20</td>
<td>3</td>
<td>8.90 ± 1.80</td>
<td>6</td>
<td>18.2 ± 1.71‡</td>
<td>3</td>
<td>4.90 ± 0.75#</td>
<td>5</td>
</tr>
<tr>
<td>Fraction of long events (%)</td>
<td>1.36 ± 0.32</td>
<td>3</td>
<td>1.35 ± 0.40</td>
<td>6</td>
<td>3.20 ± 0.47‡</td>
<td>3</td>
<td>2.46 ± 0.23‡</td>
<td>5</td>
</tr>
</tbody>
</table>

**Supplemental Table 2.** Burst gating analysis for mCa1 and mCa2 channels from failing and non-failing human hearts using two complementary detection modes. First, we used the one-burst-in-sweep detector (OBIS): A burst was defined as the interval between the first opening and the last closure in a sweep. The last opening in such burst sequences was excluded from the calculation of burst open durations. Secondly, a channel burst was considered in the presence of long open events (long-event detector; LE):¹ (i) Single and isolated openings with open durations ≥ (5 x mean open time) and/or (ii) sequences of openings with sequence open durations ≥ (5 x mean open time) were interpreted as long open events, with a sequence of openings containing a maximum of three openings and isolated openings being separated by less than three closed data points of 0.1ms length (which is our data time resolution). All ultra-short closed events were included in the duration for the sequence of long openings.⁵ Given this analysis the fraction of long events (%LE), i.e. the ratio of long events to the total number of events multiplied by 100, was considered the most reliable parameter to describe bursting.

*p<0.05 vs. respective non-failing, †p<0.05 vs. mCa1 non-failing, ‡p<0.05 vs. mCa1 failing.
**Supplemental Table 3**

<table>
<thead>
<tr>
<th>mCa1 (TP-100 mV)</th>
<th>probability (%)</th>
<th>level k = 1</th>
<th>level k = 2</th>
<th>level k = 3</th>
<th>level k = 4</th>
<th>level K = 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Failing</strong></td>
<td>P&lt;sub&gt;bin&lt;/sub&gt;</td>
<td>2.0</td>
<td>0.55</td>
<td>0.19</td>
<td>0.09</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>P&lt;sub&gt;o&lt;/sub&gt;</td>
<td>2.2</td>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
</tr>
<tr>
<td>Binomial P(n=k)</td>
<td>3.1</td>
<td>0.09</td>
<td>3.0·10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>9.4·10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>2.3·10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Poisson P(n=k)</td>
<td>3.0</td>
<td>0.05</td>
<td>4.0·10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>3.8·10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>&lt;10&lt;sup&gt;6&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>confidence ratio</td>
<td>n.d.</td>
<td>84</td>
<td>98</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Binomial</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>confidence ratio</td>
<td>n.d.</td>
<td>91</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Poisson</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>P&lt;sub&gt;MAX&lt;/sub&gt;&lt;sub&gt;N&lt;/sub&gt;</td>
<td>97</td>
<td>4</td>
<td>0.04</td>
<td>4.6·10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>5.1·10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>P&lt;sub&gt;MAX&lt;/sub&gt;&lt;sub&gt;S&lt;/sub&gt;</td>
<td>91</td>
<td>0.07</td>
<td>&lt;10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>&lt;10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>&lt;10&lt;sup&gt;6&lt;/sup&gt;</td>
<td></td>
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<tr>
<td><strong>Non-Failing</strong></td>
<td>P&lt;sub&gt;bin&lt;/sub&gt;</td>
<td>4.6</td>
<td>0.72</td>
<td>0.016</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>P&lt;sub&gt;o&lt;/sub&gt;</td>
<td>4.1</td>
<td>2.70</td>
<td>0.3</td>
<td></td>
<td></td>
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<tr>
<td>Binomial P(n=k)</td>
<td>5.4</td>
<td>0.29</td>
<td>0.015</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Poisson P(n=k)</td>
<td>5.1</td>
<td>0.14</td>
<td>0.002</td>
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<td></td>
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<tr>
<td>confidence ratio</td>
<td>n.d.</td>
<td>60</td>
<td>1&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Binomial</td>
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<td></td>
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<tr>
<td>confidence ratio</td>
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<td>88</td>
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<tr>
<td>Poisson</td>
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<td></td>
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<tr>
<td>P&lt;sub&gt;MAX&lt;/sub&gt;&lt;sub&gt;N&lt;/sub&gt;</td>
<td>95</td>
<td>8</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P&lt;sub&gt;MAX&lt;/sub&gt;&lt;sub&gt;S&lt;/sub&gt;</td>
<td>90</td>
<td>0.3</td>
<td>&lt;10&lt;sup&gt;6&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Supplemental Table 3.** Comparison between calculated and observed frequencies of simultaneous openings of channels. The kinetic parameters of pooled datasets (n=3-6) were used for stochastic calculations and comparisons. *n*: number of assumed channels; *k*: k-fold multiple stacked openings; *P<sub>bin</sub>* (P<sub>bin</sub> = \[ \frac{\text{frequency}_{k} \sum_{k} \text{dwell}_{\text{open}}}{\text{availability} \cdot M \cdot t_{\text{pulse}}} \cdot 100 < P_{o} \]): observed relative
frequency of $k$ simultaneously open levels transformed to a time-dependent probability; $P_o$: observed open probability of $k$ simultaneously open levels; $P(n=k)$: stochastic probability for $k$ simultaneous openings of $n$ assumed available channels which is given by both the binomial probability $B(n,P_o,k=n)$ and the Poisson probability $P(P_o,k)$. The confidence ratio is defined by $1 - (P(n=k)/P_{bin})$ and is an arbitrary measure not to have stacked openings due to multiple channels which open simultaneously. By high confidence ratios it is proven that mCa1 channels consists of multi-sublevels from one single channel so that the different open levels are not caused by multiple openings of more than one channel. $P_{MAX, N}$: probability to have $n=k$ simultaneous channels based on the mean open probability for one channel, $P_{MAX, S}$: probability to have one main level $k=1$ of one single channel and sublevels $k>1$ connected to this main channel. Note that the $P_{MAX}$ values obey a decreasing order: $P_{MAX, N}(k) >> P_{MAX, N}(k+1), P_{MAX, S}(i) >> P_{MAX, S}(k+1)$. In this case the combinations of $n=3$, $k=2$ and $n=3$, $k=1$ for $B(n,P_o,k=n)$ were tested; for both tests the confidence ratios were then 97% and 100%, respectively. From this result multiple openings can also be clearly excluded.
**Supplemental Table 4.** Gating parameters of $I_{mCa_1}$ and $I_{mCa_2}$ from non-failing versus failing hearts in the presence of specific drugs in bath and pipette solution (cyclosporin A [10 µM], dantrolene [10 µM], xestospongin C [10 µM], CGP-37157 [10 µM], S(-)-BayK 8644 [10 µM]). Holding potential -40 mV, test potential -100 mV. $I_{peak}$ was measured from ensemble average currents (see methods). *p<0.05 vs. respective non-failing, #p<0.05 vs. mCa1 non-failing, ‡p<0.05 vs. mCa1 failing.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>mCa1 non-failing</th>
<th>mCa1 failing</th>
<th>mCa2 non-failing</th>
<th>mCa2 failing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open probability (%)</td>
<td>6.02 ± 0.89</td>
<td>3</td>
<td>3.30 ± 0.51*</td>
<td>4</td>
</tr>
<tr>
<td>Availability (%)</td>
<td>45.4 ± 5.30</td>
<td>3</td>
<td>25.1 ± 1.21*</td>
<td>4</td>
</tr>
<tr>
<td>Mean open time (ms)</td>
<td>0.28 ± 0.03</td>
<td>3</td>
<td>0.24 ± 0.02</td>
<td>4</td>
</tr>
<tr>
<td>Mean closed time (ms)</td>
<td>4.20 ± 0.81</td>
<td>3</td>
<td>5.42 ± 0.56</td>
<td>4</td>
</tr>
<tr>
<td>Mean first latency (ms)</td>
<td>32.6 ± 2.76</td>
<td>3</td>
<td>60.1 ± 5.38*</td>
<td>4</td>
</tr>
<tr>
<td>Amplitude (pA)</td>
<td>-1.41 ± 0.06</td>
<td>3</td>
<td>-1.02 ± 0.03*</td>
<td>4</td>
</tr>
<tr>
<td>$I_{peak}$ (fA)</td>
<td>-56 ± 9</td>
<td>3</td>
<td>-15 ± 4*</td>
<td>4</td>
</tr>
<tr>
<td>Conductance (pS)</td>
<td>14.1 ± 0.40</td>
<td>3</td>
<td>10.0 ± 0.61*</td>
<td>4</td>
</tr>
</tbody>
</table>
Supplemental Figure 1

Supplemental Figure 1. Open- and closed-time histograms for $I_{\text{mCa1}}$ (A/B) and $I_{\text{mCa2}}$ (C/D) at $V_m =-100$ mV. All $\tau$-values ($\tau_o$, $\tau_c$) were obtained from the best-fitted curves by a simplex maximum likelihood estimation (judged by LLR ratios). The life-times of the open-states are longer for non-failing mitochondrial Ca$^{2+}$ channels than for failing $I_{\text{mCa1/2}}$, whereas the life-times of the closed states are longer for failing $I_{\text{mCa1/2}}$ at the same test potential ($n=3-6$).
Supplemental References


