**SUPPLEMENTAL FIGURES**

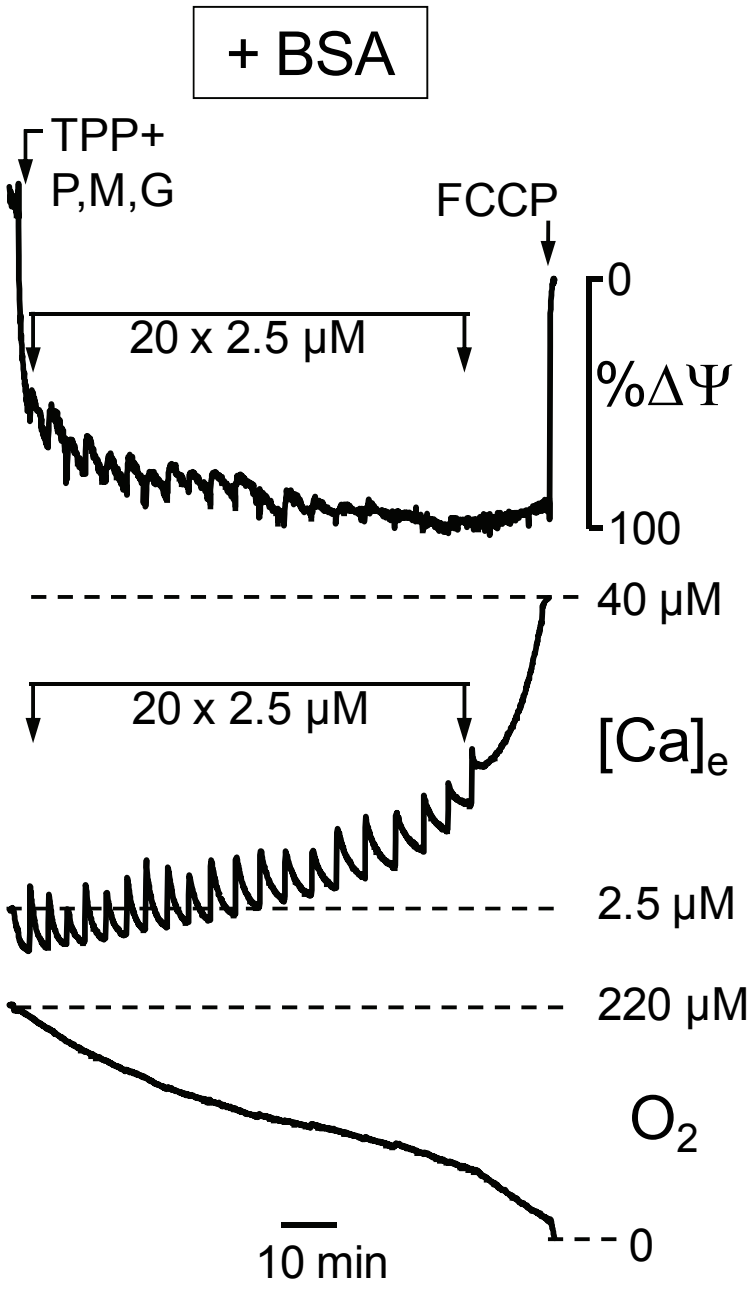


**Fig. S1.** Ca loading (20 pulses of 2.5 µM each) in KCl buffer the presence of BSA induces matrix Ca release with maintained $\Delta\psi_m$, as measured with a $TPP^+$ electrode to record $\Delta\psi_m$ (upper trace), Ca Green-5N to record free [Ca] in the buffer (middle trace), and an $O_2$ electrode to record buffer [$O_2$] (lower trace). Note that $O_2$ consumption increased significantly during matrix Ca release demonstrating that respiratory chain activity remained fully intact, consistent with transient mPTP opening in a low conductance mode, but not a high conductance mode (which would deplete matrix metabolites required for respiratory chain acceleration).

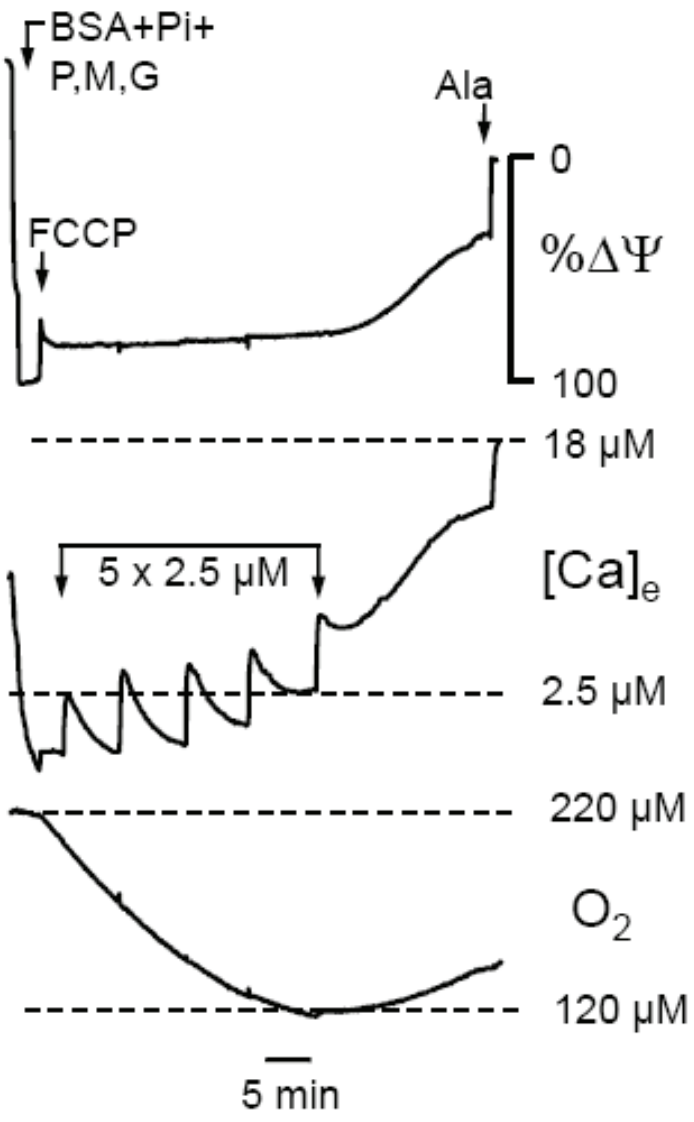


**Fig. S2.** With BSA present, a low FCCP concentration (25 nM) abrogates $\Delta\psi_m$ maintenance during matrix Ca release. Mitochondria were added to KCl buffer containing Pi (2.5 mM), pyruvate, malate, glutamate (p,m,g) and BSA (1 mg/ml). FCCP addition caused mild depolarization (upper trace, measured with TMRM). Only 5 Ca pulses (2.5 μM each) were required to induce matrix Ca release (middle trace, measured with a Ca electrode). Note that $O_2$ consumption (lower trace) decreased after matrix Ca release, indicating loss of respiratory chain power, consistent with long-lasting mPTP opening depleting matrix metabolites.

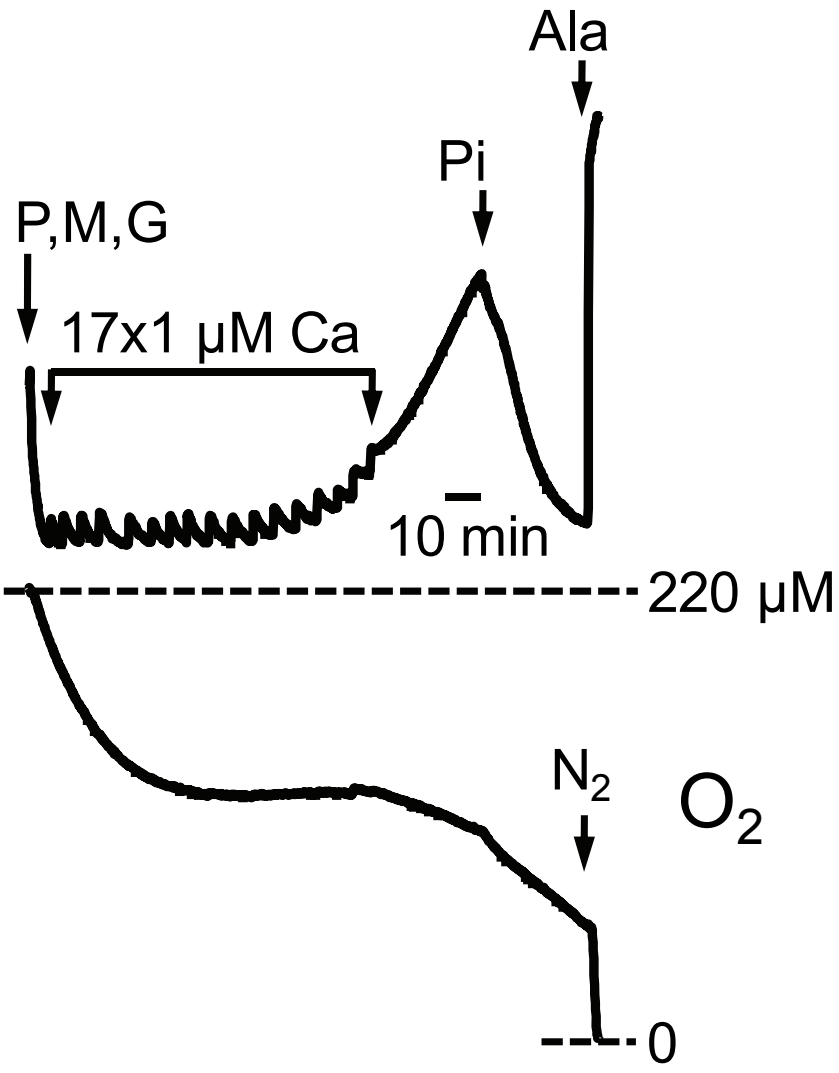


**Fig. S3.** Mitochondria added to KCl buffer containing complex I substrates (P,M, G), 0.8 µM Ca Green 5N, and BSA 1mg/ml were challenged with Ca pulses (1µM) to induce matrix Ca release. Addition of 2.5 mM Pi induced Ca reuptake. Alamethicin was added at the end to release matrix Ca, and the cuvette equilibrated with $N_2$ to verify the zero level of $O_2$.

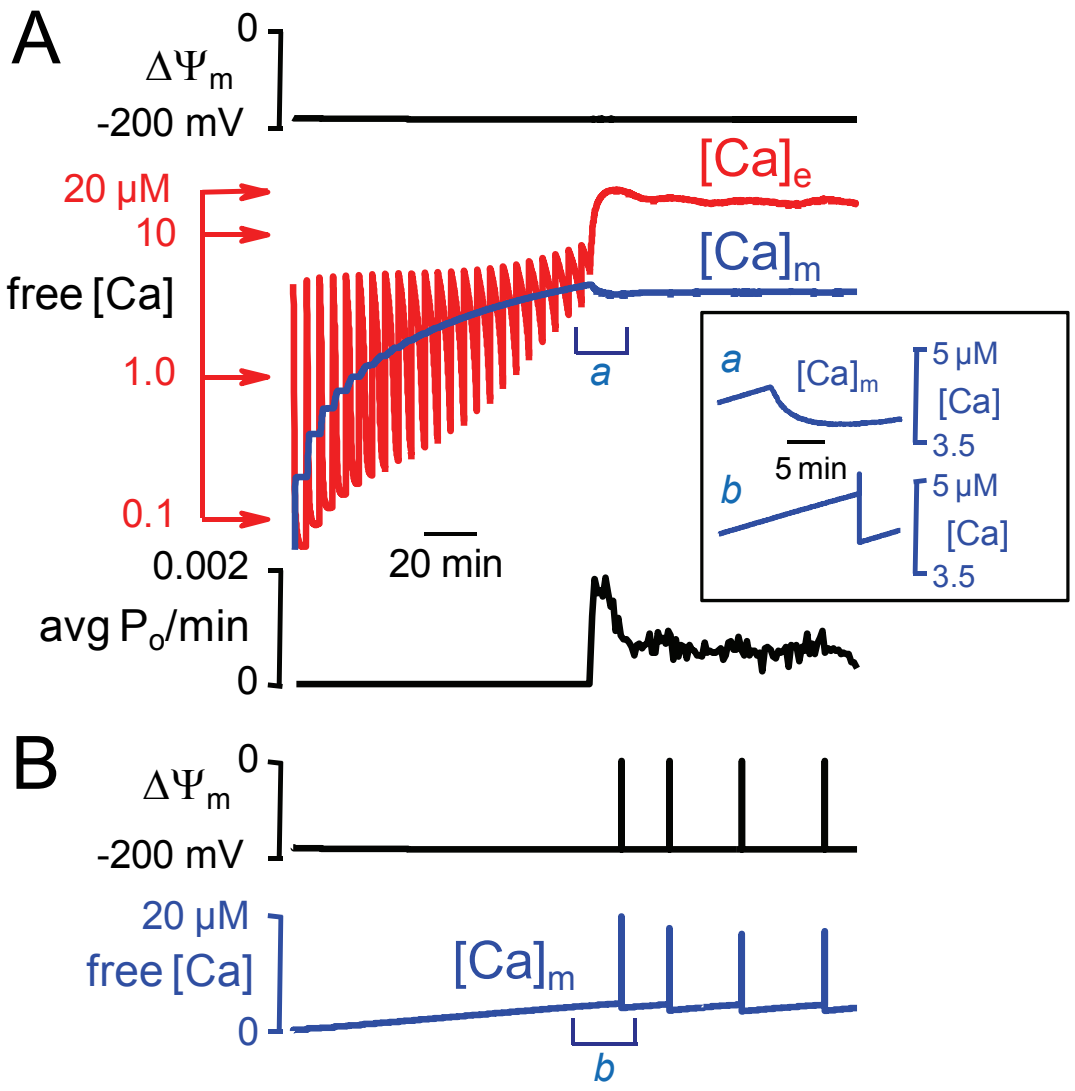


**Fig. S4. Computational model simulating transient mPTP openings which allow matrix Ca release without collective $\Delta\Psi_m$ dissipation.** **A.** Simulation of 2,000 mitochondria, each with stochastic mPTP openings as a function of matrix free [Ca] ($[Ca]_m$). Average $\Delta\Psi_m$ (*upper black trace*) is well-maintained as successive Ca pulses are added, even as extramitochondrial free [Ca] ($[Ca]_e$, *red trace*) rises to 50 μM due to random, transient mPTP openings when $[Ca]_m$ (*blue*) reaches ~5 μM. Average mPTP open probability (*lower black trace*) shows that <0.2% of mitochondria are depolarized at any given time. **B.** Time course of changes in $\Delta\Psi_m$ (*black*) and $[Ca]_m$ (*blue*) in a representative mitochondrion. $\Delta\Psi_m$ depolarizes only briefly, whereas the matrix Ca refills slowly with a half-time of several minutes. Inset to the left shows the average and individual $[Ca]_m$ on an expanded scale, just before and after matrix Ca release (corresponding to regions labeled *a* and *b* in the $[Ca]_m$ traces).

In the model, each mitochondrion containing a single mPTP which opened stochastically when matrix free [Ca] exceeded 5 μM (1). Assuming mPTP single channel conductance of 1.3 nS, an upper limit matrix volume of $10^{-12}$ liters/mitochondrion (i.e. 1 $\mu m^3$), and matrix Ca buffering of 5,000:1 (2, 3), we estimated the time constant required for matrix Ca to reequilibrate from 5 μM to <

1 μM when a single mPTP opened to be <20 ms (see below). We therefore tuned the transient mPTP opening in the model to last ~300 ms. In the simulation, an individual mitochondria needed to open only about once every 25 min to flush matrix Ca. This yielded a ratio between rates of release and reuptake of 5,000:1, so that for all 2,000 mitochondria, the average mPTP open probability was only ~0.0002. Thus, as long as mPTP openings occurred asynchronously, only one of 2,000 mitochondria was depolarized 40% of the time on average. Note also that in the simulation, mPTP openings led to net matrix Ca release even when extramitochondrial Ca had risen to 20 μM. This was attributed to the reduction in matrix Ca buffering capacity by reduced pH due to rapid proton equilibration when mPTP were open (4, 5). Thus, when mPTP opening acidifies the matrix, Ca released from the buffer transiently drives matrix free Ca to levels in excess of 20 μM, promoting net efflux. When the mPTP closes, the matrix is rapidly re-alkalinized, increasing matrix buffering capacity and rapidly lowering matrix free Ca to <1 μM.

In summary, this computational model substantiates a quantitatively plausible mechanism by which cardiac mitochondria can use transient mPTP openings to depolarize briefly, flush matrix Ca, and then rapidly repolarize (assuming essential matrix metabolites such as NADH have been retained) to return to their essential role of ATP synthesis in the face of greatly elevated extramitochondrial [Ca].

## SUPPLEMENTAL METHODS

### Experimental isolated mitochondrial studies

*Mitochondrial membrane potential* ($\Delta\psi_m$): Tetramethylrhodamine methyl ester (TMRM, 400 nM) was included in the cuvette solution, and $\Delta\psi_m$ estimated from TMRM fluorescence at 580 nm ($F_{TMRM}$) as described previously (6). $\Delta\psi_m$ is expressed as percentage of the TMRM fluorescence in the presence of coupled mitochondria and substrates, where 100% refers to the $F_{TMRM}$ value immediately after the addition of mitochondria to the cuvette, and 0% to the $F_{TMRM}$ value after addition of 0.5 μM FCCP or alamethicin (5 μM) to fully depolarize mitochondria at the end of the tracing. Alternatively, $\Delta\psi_m$ was monitored by using a $TPP^+$ selective electrode in the presence of 1.5-3.0 μM $TPP^+$.

*Mitochondrial Ca uptake and efflux:* Changes in extramitochondrial Ca were followed using a Ca-sensitive electrode. Free [Ca] was calibrated at two time points in each experiment. The first time point was the peak response of the Ca electrode to the first Ca pulse (typically 2 or 2.5 μM) after mitochondria were added to the buffer, assuming that the free [Ca] before the Ca pulse was <0.2 μM. The second time point was the final reading of the Ca electrode after alamethicin (5 μM) was added to the buffer to release accumulated matrix Ca. This reading was assumed to represent 5 μM (the measured free [Ca] of the buffer in the absence of mitochondria) plus the total amount of Ca added during the experiment. As control, we established that when no additional Ca was added to mitochondria, alamethicin did not significantly alter the Ca electrode reading, indicating that isolated mitochondria contained an insignificant amount of alamethicin-releasable Ca relative to the 5 μM free [Ca] in the buffer. In some experiments, extramitochondrial free Ca was estimated by adding Calcium Green-5N (1 μM, salt form) to the buffer, and recording fluorescence at excitation/ emission wavelengths of 490/530 nm.

*Mitochondrial $H_2O_2$ production:* To measure mitochondrial $H_2O_2$ production, Amplex Red (10 μM) + 0.2U horseradish peroxidase was added directly to the buffer (containing 0.2 mM Pi) and the increase

in Resorufin fluorescence (excitation/emission 540 nm/590 nm) proportional to $H_2O_2$ production was recorded.

*Mitochondrial swelling.* Changes in matrix volume were monitored by light scattering at 540 nm.

*Mitochondrial protein:* Protein content was determined by the Lowry method.

**Mathematical modeling**

We simulated a population of 2,000 mitochondria, with the $\Delta\Psi_m$ of each mitochondrion governed by a differential equations similar to previous studies (7, 8). We assumed one mPTP per mitochondrion, simulated using a Markovian model with stochastic opening and closing. During the MPTP opening, the mitochondrion is fully depolarized allowing Ca to rapidly exit the matrix by flowing down its concentration gradient. $\Delta\Psi_m$ of the whole system is simply the average of the individual mitochondrial potentials, i.e., $\Delta\Psi_m = \sum_{i=1}^{2000} \Delta\Psi_m^i / 2000$. The details of the modeling and computer simulation are described below.

**1. Model**

*Mitochondrial membrane potential ($\Delta\Psi_m$) and matrix Ca ($Ca_m$) of a single mitochondrion*. The governing differential equation for $\Delta\Psi_m$ of an isolated mitochondrion was adopted from previous studies (7, 8) with modifications, i.e.,

$$\frac{d\Delta\Psi_m}{dt} = \frac{V_{He} + V_{He(F)} - V_{Hu} - V_{ANT} - V_{HLeak} - V_{NaCa} - 2V_{up} - V_{MPTP}}{C_{mito}} \qquad (1)$$

where *V's* are the rates of different processes that contribute to $\Delta\Psi_m$ as described in detail in the work of Cortassa et al (7), except for $V_{MPTP}$. $V_{MPTP}$ is the rate of $\Delta\Psi_m$ dissipation due to the mitochondrial permeability transition pore (mPTP) opening, which is simply formulated as $V_{MPTP} = G_M P(t)\Delta\Psi_m$ where $P$ is the state of the mPTP, and $P(t)$=1 when mPTP opens and $P(t)$=0 when mPTP closes. Here we assume one mPTP per mitochondrion. The differential equation for $Ca_m$ is:

$$\beta \frac{dCa_m}{dt} = \frac{V_{up} - V_{NaCa}}{2v_m F} - J_{MPTP} / v_m \qquad (2)$$

where $v_m$ is volume of the mitochondrion, $\beta$ is the $Ca_m$ buffering coefficient, $F$ is the Farady constant, and $J_{MPTP}$ is the Ca flux through the mPTP modeled as $J_{MPTP} = G_{M,Ca} P(t)(\Delta\Psi_m - \frac{RT}{F}\ln\frac{Ca_m}{Ca_c})$, where $G_{M,Ca}$ is the Ca conductance of the mPTP and $Ca_c$ is the Ca concentration outside the matrix. Mitochondrial Na/Ca exchange is modeled by Ca efflux through a Na/Ca antiporter (Cortassa et al., 2003). Since we ignore the Na concentration in our model, the exchange flux is simplified to be only matrix Ca dependent: $V_{NaCa} = k_{anti} Ca_m$, where the $k_{anti} = 0.3\mu M$.

*Mitochondrial Ca uptake*. Since the depolarization of $\Delta\Psi_m$ due to mPTP opening occurs for a very short time interval, while the uptake of Ca occurs on a much slower time scale, we simply

model the mitochondrial Ca uptake as a function of $Ca_c$ as: $V_{up} = V_{Max}^{uni}(0.1 + 0.9e^{-t/600})\frac{Ca_c^3}{Ca_c^3 + 0.5^3}$,

where $V_{Max}^{uni} = 42\mu M/s$ is the maximum uptake rate. Based on the experimental observation (Fig.6) that the Ca uniporter is inactivated to about 10% of its maximum value, we assumed that the uptake rate decayed exponentially with time constant 10 min (600 s).

*Stochastic opening of mPTP*. The stochastic opening of mPTP was modeled using a simple Markovian model: $C_1 \leftrightarrow C_0 \leftrightarrow O$, where $C_0$ and $C_1$ are the two closed states and O is the open state. The transition from $C_1$ to $C_0$ is Ca-dependent (Fig.A1), i.e., as free Ca accumulates in the matrix, the transition probability from $C_1$ to $C_0$ increases, and thus the opening probability of mPTP increases. The rate constants used in this study are as follows: $\alpha_0 = 0.1$, $\beta_0 = 10$, $\beta_1 = 0.01$, and $\alpha_1 = 0.0001 * [1 + 199 * \frac{e^{4Ca_m} - 1}{e^{4Ca_m} + e^{4Ca_0} - 2}]$, where $Ca_0$=5 μM is a constant.

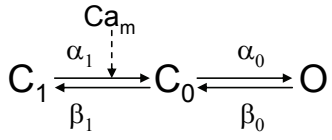

Fig.A1. The Markovian model of mPTP.

*Mitochondrial population in a cuvette*. To investigate the collective behavior of a mitochondrial population, we simulated 2,000 mitochondria coupled diffusively through the Ca concentration outside the matrix ($Ca_c$). The volume ratio between the outside space and the matrix ($\gamma$) was set as $4x10^5$. Since the diffusion rate of Ca is much faster than the Ca uptake rate, we assumed Ca distribution in the outside space to be uniform, such that the differential equation for $Ca_c$ is:

$$\frac{dCa_c}{dt} = [\sum_{i=1}^{2000}(\frac{V_{NaCa}^i - V_{up}^i}{2F} + J_{MPTP}^i)]/\gamma \qquad (3)$$

where γ is the volume ratio of the space outside the matrix and the matrix. The membrane potential of the whole mitochondria population in the cuvette is:

$$\Delta\Psi_m = \sum_{i=1}^{2000}\Delta\Psi_m^i / 2000 \qquad (4)$$

**2. Computer simulations**

Since the mPTPs open and close quickly, a very small time step is required to calculate $\Delta\Psi_m$ and $Ca_m$ of a single mitochondrion using Eqs.1 and 2 during a mPTP open and close cycle. To overcome this difficulty in simulation, $\Delta\Psi_m$ and $Ca_m$ were calculated as described below.

*Mitochondrial membrane potential loss due to mPTP open*. The mPTP is a large nonselective channel. Once the mPTP open, matrix ions with mass<1.5 kDa exit the mitochondrion, effectively short-circuiting proton-coupled energy transduction and dissipating $\Delta\Psi_m$. Therefore we assume that $\Delta\Psi_m$ is immediately depolarized to zero once an mPTP opens.

*Mitochondrial membrane potential recovery due to mPTP closing*. When the mPTP closes, the rate of repolarization is determined mainly from the term $V_{He}/C_{mito}$ (Eq.1), in which $V_{He}$ is

about 100-150 mM/s during depolarization, and $C_{mito}$=1.812 mM/V (Cortassa et al., 2003;). Therefore, $\frac{d\Delta\Psi_m}{dt} \approx \frac{V_{He}}{C_{mito}} \in (55.18, \quad 82.78)$ V/s, and the membrane potential takes only 2.2-3.3 ms to repolarize from 0 to -180 mV. Based on this estimate, we assume that $\Delta\Psi_m$ repolarizes immediately to -180 mV once the mPTP closes.

*Matrix Ca buffer.* Most matrix Ca is buffered by Pi, in a pH-sensitive manner (4, 5). We assume that buffering ratio between buffered Ca and free Ca is 5,000:1 when mPTP is closed ($\beta_c$=0.0002), and when mPTP is open the buffering efficiency falls to 1,000:1 ($\beta_o$=0.001) due to the decrease in matrix pH (4, 5). Therefore, at the moment of mPTP opening, the free matrix Ca ($Ca_m$) changes to ($Ca_m/\beta_c + Ca_m$)*(1- $\beta_o$).

*Matrix Ca loss due to mPTP openning.* To estimate the rate of matrix Ca efflux during an mPTP opening, we used the following equation:

$$\frac{dCa_m}{dt} = \frac{G_{M,Ca}(\Delta\Psi - \frac{RT}{F}\ln\frac{Ca_m}{Ca_c})}{2vF}$$

where $G_{M,Ca}$=1.3X10$^{-9}$ S(9), $RT/F$=0.027 V, $Ca_c$=0.1 μM, $F$ is the Faraday constant which is 0.0965 C/μmol, and $v$=4/3πr$^3$ is the mitochondria volume. Assume $r$=0.5 μm, then $v$=0.52 μm$^3$=0.52X10$^{-18}$ m$^3$=0.52X10$^{-15}$ l. When mPTP opens, ΔΨ=0 and then we have:

$$\frac{dCa_m}{dt} = \frac{1.3\times10^{-9}(0 - 0.027\ln\frac{Ca_m}{0.1})}{2\times0.52\times10^{-15}\times0.0965} \approx -1.3*0.027\times10^7\ln\frac{Ca_m}{0.1} = -3.5\times10^5\ln\frac{Ca_m}{0.1}.$$

Since the buffering efficacy falls substantially after a mPTP opening, the free Ca in the matrix increases sharply. To estimate the time required for total matrix Ca to fall from 10,000 μM to 0.5 μM (Cyto Ca level), $\ln\frac{Ca_m}{0.1}$ is between 1.6 and 11.5, such that -4*10$^6$ < |dCa$_m$/dt| < -5.6*10$^5$ μM/s. Thus, mPTP release rate exceeds 5.6*10$^5$ μM/s, taking less than 10,000/(5.6*10$^5$) s= 18 ms, to release 10,000 μM Ca. Based on this estimate, we assume that once mPTP opens, the matrix free Ca first changes to (Ca$_m$/β$_c$+ Ca$_m$)*(1- β$_o$) due to the pH-sensitive change in Ca buffering efficacy, such that free Ca is released instantaneously from the matrix buffer and equilibrates with the Ca outside. Therefore, after an mPTP opens in a mitochondrion, the matrix free Ca changes to

$$\frac{(\frac{Ca_m}{\beta_c} + Ca_m)(1-\beta_o) + \gamma Ca_c}{1+\gamma}$$, and the Ca in the cuvette changes to

$$Ca_c + \frac{(\frac{Ca_m}{\beta_c} + Ca_m)(1-\beta_o) - Ca_c}{1+\gamma}.$$

*Other numerical methods*. The differential equations were numerically solved using the 4$^{th}$-order Runge-Kutta. The stochastic opening and closing of the mPTP was simulated using methods developed by Gillespie (10) and Clay and DeFelice (11).

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