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Supporting Material

Mitochondrial Free [Ca^{2+}] Increases during ATP/ADP Antiport and ADP Phosphorylation: Exploration of Mechanisms

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Supporting material for "Mitochondrial Free [Ca^{2+}] Increases during ATP/ADP Antiport and ADP Phosphorylation: Exploration of Mechanisms"

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Mitochondrial isolation

All experiments conformed to the Guide for the Care and Use of Laboratory Animals, and were approved by the animal studies committee. Guinea pig heart mitochondria were isolated as described before (1,2). Briefly, guinea pigs (250-350 g) were anesthetized by intraperitoneal injection of 30 mg ketamine, and the blood was anticoagulated with 700 units of heparin. Hearts (n = 48) were excised and minced to approximately 1 mm^3 pieces in ice-cold isolation buffer containing in mM: mannitol 200, sucrose 50, KH_2PO_4 5, 3-(N-morpholino) propanesulfonic acid (MOPS) 5, EGTA 1, BSA 0.1%, pH 7.15 (adjusted with KOH). The minced heart was suspended in 2.65 ml buffer with 5U/ml protease, and homogenized at low speed for 20 s; next 17 ml isolation buffer was added and the suspension was again homogenized for 20 s. The suspension was centrifuged at 8000 *g* for 10 min. The supernatant was discarded and the pellet was resuspended in 25 ml isolation buffer and centrifuged at 900 *g* for 10 min. The supernatant was centrifuged once more at 8000 *g* to yield the final mitochondrial pellet, which was suspended in 0.5 ml isolation buffer and kept on ice. The mitochondrial protein concentration was measured using the Bradford method (3), and diluted with isolation buffer to 12.5 mg mitochondrial protein/ml.

Measurement of matrix free [Ca^{2+}].

$[Ca^{2+}]_m$ was measured in indo-1 acetoxymethyl (AM) loaded mitochondria from 16 hearts. Indo-1 is a fluorescent dye that binds to Ca^{2+} with a K_d of approximately 220 nM. The emission wavelength (λ_{em}) shifts from 456 nm to 390 nm on binding to Ca^{2+} at an excitation wavelength of 350 nm (λ_{ex}). The ratio between the two λ_{em}'s correct for differences in the amount of dye taken up into the mitochondria. Since the emission and excitation wavelengths (λ_{ex}) are the same as for NADH, the two NADH background emission signals were subtracted from the two emission indo-1 signals before calculating the ratio (R). The ratios obtained when all indo-1 had become bound to Ca^{2+} (R_{max}) and when no Ca^{2+} was bound to indo-1 (R_{min}) were measured in energized mitochondria using 500 nM cyclosporine A and 500 μM $CaCl_2$ for the R_{max} and A23187 (Ca^{2+}-ionophore) and 2.5 mM EGTA for the R_{min}. $[Ca^{2+}]_m$ was calculated using the equation (4):

$$[Ca^{2+}]_m \text{ (nM)} = K_d \times S_{f2}/S_{b2} \times (R-R_{min})/(R_{max}-R)$$

The K_d value was 220 nM, and S_{f2} is the signal intensity of free indo-1 measured at 456 nm, S_{b2} is the signal intensity of Ca^{2+}-saturated indo-1 measured at 456 nm. Their ratio was measured to be 1.35. The individual $[Ca^{2+}]_m$'s were normalized to the averaged $[Ca^{2+}]_m$ over all experiments at t = 10 s, which was calculated as 80 nM.

Measurement of extra-matrix free [Ca^{2+}]

$[Ca^{2+}]_e$ was measured using indo-1 pentapotassium salt (K-indo-1), an indo-1 form that cannot penetrate the mitochondrial membrane. Mitochondria (not incubated in indo-1 AM) from 3 hearts were suspended in the experimental buffer with added K-indo-1 (1 μM). The signal was corrected for NADH. R_{max} was obtained by adding 10 mM $CaCl_2$. R_{min} was measured in experimental buffer with 2.5 mM EGTA. The equation given above was used to calculate $[Ca^{2+}]_e$.

Measurement of mitochondrial redox state

Mitochondria from 12 hearts were used to measure NADH autofluorescence. Unlike NAD, NADH molecules have natural fluorescence properties that can be monitored (5). Therefore, an increase in the signal reflects an increase in the ratio of NADH to NAD^+, i.e., a shift to a more reduced state. The emission spectrum of NADH is broad and peaks at a λ_{em} of 456 nm at a λ_{ex} at 350 nm. To correct for differences in total NADH and NAD^+ pool sizes, the ratio of 456 over 390 nm λ_{em} was measured at a λ_{ex} of 350 nm. In addition to providing data on the mitochondrial redox state, the raw NADH data was used to correct for the background autofluorescence measured by the indo-1 fluorescence probe for $[Ca^{2+}]_m$.

Measurement of $\Delta\Psi_m$

Mitochondrial membrane potential ($\Delta\Psi_m$) was measured in indo-1 treated mitochondria from 16 hearts by adding 50 nM Rh123 (Calbiochem, San Diego, CA) to the experimental buffer. Fluorescence changes were measured at a λ_{ex} of 503 nm and a λ_{em} of 527 nm. Rh123 uptake into the mitochondrion is dependent on $\Delta\Psi_m$. As the dye is taken up, the fluorescence decreases as the dye autoquenches; therefore, a decrease in $\Delta\Psi_m$ is represented by an increase in signal. Independent and combined pilot studies were done to assure that the indo-1 and Rh123 dyes do not spectrally interfere with each other.

Measurement of matrix pH

Matrix pH was measured in 2'7'-bis-(2-carboxyethyl)-5'(and 6-) carboxy-fluroescein (BCECF) AM treated mitochondria from 9 hearts using a λ_{ex} of 504 nm and a λ_{em} of 530 nm. BCECF is a fluorescent probe that becomes less fluorescent in an acidic environment; thus an increase in signal indicates matrix alkalinization and a decrease in signal indicates matrix acidification. The measured signals were normalized for each group to their average photon count at the steady state observed after adding $CaCl_2$ or vehicle to correct for differences in signal strength and dye uptake. The measured signal was converted to pH units by measuring the BCECF signal from tritonized (1% triton X-100) mitochondria, which were incubated in BCECF in buffers with known pH (7.00, 7.15 and 7.25). This gave a linear slope, which enabled calculation of matrix pH from the signal intensity. Because the wavelengths used for BCECF measurements do not interfere with the NADH autofluorescence signals, matrix NADH and pH measurements were conducted in the same mitochondrial preparation.

Measurement of extra-matrix Mg^{2+}

Mitochondrial buffer [Mg^{2+}] was assessed in 12 experiments by the reaction of Mg^{2+} with methylthymol blue (MTB) to form a blue complex that is proportional to [Mg^{2+}] and which was measured using bichromatic 600 and 510 nm endpoints (Siemens Healthcare Diagnostics, Inc., Newark, DE). Interference by buffer Ca^{2+} was eliminated by the presence of Ba-EGTA. Buffer [Mg^{2+}] was 0.54±0.08 mM (SEM) without added $MgCl_2$; after addition of 1 mM $MgCl_2$ to the experimental buffer, [Mg^{2+}] rose to 0.84±0.02 mM. In some experiments mitochondria were lysed with Triton-X and the lysate-buffer [Mg^{2+}] was measured. Using a mitochondrial density estimate of 1300 mg/ml (minus H_2O = 300 mg/ml mitochondria) and a mitochondrial protein

concentration of 0.5 mg/ml, this gave a mitochondrial volume to buffer volume ratio of approximately 1:600. Calculated mitochondrial matrix [Mg^{2+}] was 0.51±0.03 mM without added $MgCl_2$ and 0.85±0.02 mM after addition of 1 mM $MgCl_2$ to the buffer.

Measurement of mitochondrial volume

Mitochondrial matrix volume was assessed by the light scattering technique (6) at a λ520 nm in 6 hearts. Mitochondria were energized with PA and exposed to increases in buffer $CaCl_2$ and 250 μM ADP as in other protocols. Maximal volume expansion was assessed using 10 nM valinomycin, a K^+ specific ionophore.

Measurement of mitochondrial respiration

O_2 consumption was measured using a Clark-2 O_2 electrode at 25ºC. Mitochondria (0.5 mg protein/ml) isolated from 6 hearts were suspended in experimental buffer. PA (0.5 mM) was added to initiate state 2 respiration; this was followed by addition of [$CaCl_2$] (0, 10 or 25 μM). States 3 and 4 respiration were measured after addition of exogenous ADP (250 μM) and consumption of ADP, respectively. The ratio of slopes of O_2 consumption during state 3 and state 4 respirations was calculated to determine the respiratory control index (RCI).

Table A. Dissociation constants (K_d's) of ADP^{3-}, ATP^{4-} and P_i^- for Ca^{2+}, Mg^{2+}, H^+ and K^+ at 25ºC, at an ionic strength of 0.17 M.

K_d	Ca^{2+}	Mg^{2+}	H^+	K^+
P_i^-	34.4 mM	20.4 mM	0.20 μM	1017 mM
ATP^{4-}	0.28 mM	0.15 mM	0.26 μM	13.5 mM
ADP^{3-}	2.49 mM	1.62 mM	0.38 μM	25.5 mM

K_d's were obtained from the National Institute of Standards and Technology (NIST).

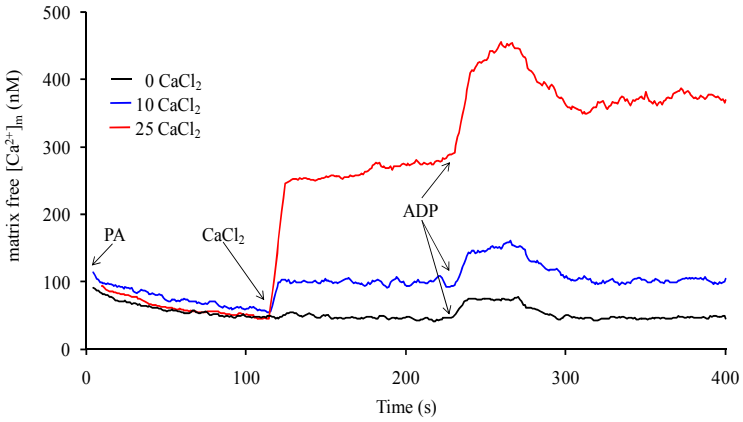

Fig. S1 *A*. Responses to added $CaCl_2$ and ADP in the presence of 1 mM $MgCl_2$. Note that $[Ca^{2+}]_m$ increased less with added $CaCl_2$ when $MgCl_2$ was added to the buffer (compare with Fig. 3A) but the proportional increase in $[Ca^{2+}]_m$ when 250 µM ADP was added was similar to that observed in the absence of added $MgCl_2$ (compare with Fig. 3A).

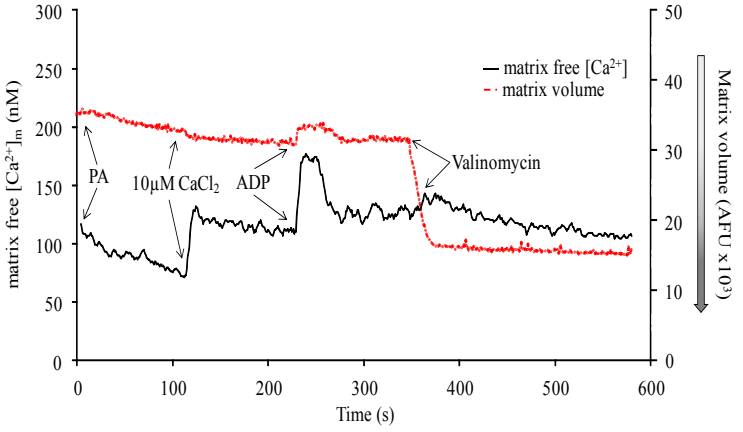

Fig. S1 *B*. Simultaneous changes in $[Ca^{2+}]_m$ and matrix volume on addition of 10 mM $CaCl_2$, 250 µM ADP, and 10 nM valinomycin in energized mitochondria. Note that matrix volume contraction on addition on ADP was very small compared to the matrix volume expansion after valinomycin. Condition: PA: 0.5 mM pyruvic acid.

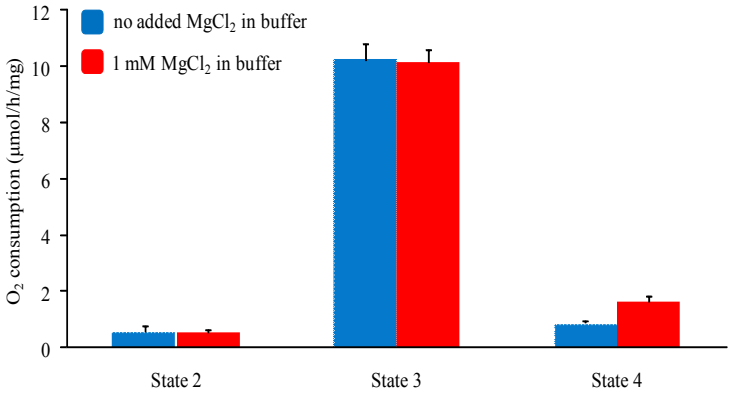

Fig. S1 *C*. Rates of mitochondrial respiration with and without added 1 mM $MgCl_2$ before (state 2), during 250 µM ADP addition (state 3), and after ADP phosphorylation (state 4). Note that adding $MgCl_2$ did not alter respiration rates. Conditions: 0.5 mM pyruvic acid, no added $CaCl_2$.

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