**F-ATPase of Drosophila melanogaster Forms 53-Picosiemen (53-pS) Channels Responsible for Mitochondrial Ca\(^{2+}\)-induced Ca\(^{2+}\) Release**

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**Background:** The Ca\(^{2+}\)-induced Ca\(^{2+}\) release channel (mCrC) of *Drosophila* mitochondria is similar to the permeability transition pore (PTP).

**Results:** mCrC is modulated by PTP effectors and *Drosophila* F-ATPase forms 53-pS channels.

**Conclusion:** F-ATPase mediates Ca\(^{2+}\)-induced Ca\(^{2+}\) release in *Drosophila* mitochondria.

**Significance:** Channel formation by F-ATPases has been conserved in evolution, but species-specific features exist that may underscore different roles in different organisms.

Mitochondria of *Drosophila melanogaster* undergo Ca\(^{2+}\)-induced Ca\(^{2+}\) release through a putative channel (mCrC) that has several regulatory features of the permeability transition pore (PTP). The PTP is an inner membrane channel that forms from F-ATPase, possessing a conductance of 500 picosiemens (pS) in mammals and of 300 pS in yeast. In contrast to the PTP, the mCrC of *Drosophila* is not permeable to sucrose and appears to be selective for Ca\(^{2+}\) and H\(^+\). We show (i) that like the PTP, the mCrC is affected by the sense of rotation of F-ATPase, possessing a conductance of 500 picosiemens (pS) in yeast mitochondria (7, 8) where Pi has an inhibitory effect and F-ATPase forms from the F-ATPase under conditions of oxidative stress (6). The PTP is permeable to sucrose, as is the pore of yeast mitochondria (7, 8) where Pi has an inhibitory effect and F-ATPase forms 300-pS channels (9). Also in *D. melanogaster*, Ca\(^{2+}\)-induced Ca\(^{2+}\) release channel (mCrC, which like in yeast is inhibited rather than activated by P\(_i\)) is impermeable to sucrose, suggesting that its size may be considerably smaller than that of the PTP (1).

The recent demonstration that the PTP forms from the F-ATPase (6, 9) provides a new framework to analyze the nature of the mCrC of *D. melanogaster*. Here we have assessed whether F-ATPase dimers purified from *Drosophila* mitochondria possess channel activity. Our findings provide novel information on the channel function of F-ATPases, establish that the mCrC is the PTP of *D. melanogaster*, and shed new light on its possible role in regulation of Ca\(^{2+}\) homeostasis (10, 11).

**EXPERIMENTAL PROCEDURES**

**Cell Cultures—** *Drosophila S\(_2\)R\(^+\) cells* (12, 13) were cultured in Schneider’s insect medium (Life Technologies) supplemented with 10% heat-inactivated FBS (Life Technologies) and kept at 25 °C. Culture medium for the transected S\(_2\)R\(^+\) pActCyPD-HA/pCoPuro cells was supplemented with 8 μg/ml puromycin.

**Subcellular Fractionation—** Cells were lysed in a medium containing 10 mM Tris-HCl, pH 6.7, 10 mM KCl, 150 μM MgCl\(_2\) supplemented with protease and phosphatase inhibitor cocktails (Sigma) for 30 min on ice, followed by passage through a 26-gauge × 0.5-inch syringe (Artisana). Sucrose was then added at a final concentration of 250 mM, and lysates were centrifuged three times at 2,200 × g for 10 min at 4 °C to remove nuclei and cell debris. Mitochondria were then sedimented at 8,200 × g for 10 min at 4 °C.

**Cell Permeabilization—** Cells were detached with a sterile cell scraper, centrifuged at 200 × g for 10 min, and washed twice on unique structural features that may underscore specific roles in different species.

Mitochondria of *Drosophila melanogaster* possess an array of Ca\(^{2+}\) transport pathways, i.e. the Ca\(^{2+}\) uniporter MCU, the Na\(^+\)/Ca\(^{2+}\) exchanger NCLX, and a Na\(^+\)-insensitive Ca\(^{2+}\) efflux system (1), that display the same features as those observed in mammalian mitochondria (2–5). An important difference, however, exists.

In mammalian mitochondria, Ca\(^{2+}\) and P\(_i\) induce opening of the permeability transition pore (PTP),\(^2\) a 500-pS channel that forms from the F-ATPase under conditions of oxidative stress (6). The PTP is permeable to sucrose, as is the pore of yeast mitochondria (7, 8) where P\(_i\) has an inhibitory effect and F-ATPase forms 300-pS channels (9). Also in *D. melanogaster*, Ca\(^{2+}\)-induced Ca\(^{2+}\) release channel (mCrC, which like in yeast is inhibited rather than activated by P\(_i\)) is impermeable to sucrose, suggesting that its size may be considerably smaller than that of the PTP (1).

The recent demonstration that the PTP forms from the F-ATPase (6, 9) provides a new framework to analyze the nature of the mCrC of *D. melanogaster*. Here we have assessed whether F-ATPase dimers purified from *Drosophila* mitochondria possess channel activity. Our findings provide novel information on the channel function of F-ATPases, establish that the mCrC is the PTP of *D. melanogaster*, and shed new light on its possible role in regulation of Ca\(^{2+}\) homeostasis (10, 11).

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2 The abbreviations used are: PTP, permeability transition pore; pS, picosiemen(s); mCrC, mitochondrial Ca\(^{2+}\) release channel; BN-PAGE, blue native polyacrylamide gel electrophoresis; CyP, cyclophilin; CyPD, cyclophilin D; TOM20, translocase of outer mitochondrial membranes of 20 kDa; CsA, cyclosporin A; MCU, mitochondrial Ca\(^{2+}\) uniporter; CRC, Ca\(^{2+}\) retention capacity; OSCP, oligomycin sensitivity conferral protein; ctrl, control.

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with 130 mM KCl, 10 mM Mops-Tris, pH 7.4 (KCl medium) containing 10 μM EGTA-Tris. The resulting pellet was resuspended in KCl medium containing 150 μM digitonin and 1 mM EGTA-Tris and incubated for 20 min on ice (6 × 10^7 cells × ml^-1). Cells were then diluted 1:5 in KCl medium containing 10 μM EGTA-Tris and centrifuged at 200 g for 25 min at 4 °C. Mitochondria were suspended at 10 mg g^-1 in 1× native PAGE sample buffer (Invitrogen) supplemented with protease inhibitor mixture (Sigma), solubilized with 2% (w/v) digitonin, and immediately centrifuged at 100,000 g for 25 min at 4 °C. The supernatants were solubilized in KCl medium containing 100 mM NaCl, 20 mM Tris, pH 7.4, 5 mM EDTA-Tris at 4 °C, and the supernatants were solubilized in Laemmli gel sample buffer. For separation of F-ATPase dimers, the Laemmli buffer was directly added to the dimer bands eluted from a BN-PAGE gel. Samples were separated by 15% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes using an SE400 vertical electrophoresis unit (Hoefer). Western blotting was performed in PBS containing 3% nonfat dry milk with polyclonal goat anti-actin (Santa Cruz Biotechnology), polyclonal rabbit anti-caspase-3 (Cell Signaling), monoclonal mouse anti-CyP-D (Calbiochem), monoclonal mouse anti-HA (Sigma), monoclonal mouse anti-F-ATPase subunit β (Abcam), polyclonal rabbit anti-MCU (Sigma), or polyclonal rabbit anti-TOM20 (Santa Cruz Biotechnology) antibodies.

**BN-PAGE**—Pellets of mitochondria isolated from adult white^1118^ flies were suspended at 10 mg g^-1 in native PAGE sample buffer (Invitrogen) supplemented with protease inhibitor mixture (Sigma), solubilized with 2% (w/v) digitonin, and immediately centrifuged at 100,000 g for 25 min at 4 °C. The supernatants were solubilized with native PAGE 5% G-250 sample additive (Invitrogen) and quickly loaded onto a blue native polyacrylamide 3–12% gradient gel (Invitrogen). After electrophoresis, gels were processed, and F-ATPase dimers were prepared for bilayer experiments exactly as described (9).

**Immunofluorescence**—One day before the experiments, stably transfected S2R^pActCyPD-HA cells were seeded on sterilized 13-mm round glass coverslips in a 24-well tissue culture plate at 2 × 10^5 cells/well in 0.5 ml of culture medium. On the day of the experiment, cells were washed once with PBS and incubated for 20 min at room temperature with 0.5 ml of serum-free Schneider’s medium supplemented with 1 μg/ml CsH and 100 nM MitoTracker Red CMXRos (Molecular Probes). Cells were fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 50 mM NH4Cl in PBS + 0.1% Triton X-100 for 5 min at room temperature, and blocked with PBS containing 3% goat serum for 1 h at room temperature with PBS washes between each step. Monoclonal anti-HA (clone HA-7, Sigma) in PBS with 2% goat serum was added, and incubation was carried out overnight at 4 °C. On the next day, cells were washed with PBS, and the immunoreaction was revealed with FITC-conjugated anti-mouse IgG (Fab-specific, Sigma) in PBS with 2% goat serum for 45 min at room temperature. Coverslips were examined with an Olympus epifluorescence microscope at 60× magnification.

**Cell Transfection, Plasmids, and Constructs**—A construct of human cyclophilin (CyP) D cDNA carrying a N-terminal mitochondrial targeting sequence from *Drosophila* Hsp60 (a mitochondrial matrix protein) and a C-terminal HA tag was generated by PCR using total cDNA of the human osteosarcoma cell line HQB17 as template. Oligonucleotides were designed using the on-line tool Primer-BLAST (16). Primer sequences were 5’-ctgttgacctatgtttccagtttgcttggctctctctactatgaagcaggccagttgccaagcggcagatg-3’ (forward) and 5’-ccagctcaatgtaagcttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttctagggggttt
**REPORT: Drosophila F-ATPase Forms 53-pS Channels**

*sophila actin 5c* promoter (a gift of M. F. Ceriani, Laboratorio de Genética del Comportamiento, Fundación Instituto Leloir and Instituto de Investigaciones Bioquímicas, Buenos Aires, Argentina) co-transfected with the selection vector pCoPuro (Addgene plasmid 17533), generated by Dr. Francis Castellino (17). Two million cells/well were plated in a 6-well tissue culture plate in 2 ml of culture medium/well. Cells were incubated for 5 h at room temperature and then transfected with the Effectene transfection reagent kit (Qiagen) with a ratio of selection vector (pCoPuro) to expression vector (pActCyPD-HA) of 1:20. After 3 days, the medium was replaced with culture medium containing 8 μg/ml puromycin for selection.

**Electrophysiology**—Planar lipid bilayer experiments were performed as described in Ref. 18. Briefly, bilayers of 150–200 picofarads capacitance were prepared using purified soybean asolectin. The standard experimental medium was 100 mM KCl, 10 mM HEPES, pH 7.4. All reported voltages refer to the cis chamber, zero being assigned to the trans (grounded) side. Currents were considered as positive when carried by cations flowing from the cis to the trans compartment. Freshly prepared F-ATPase dimers were added to the cis side. No current was observed when PTP activators were added to the membrane in the absence of F-ATPase dimers. The i-V curve was obtained in the range of ~80 to +40 mV from three independent experiments and contained current values that were measured manually (n > 30 for each potential) using the PCLAMP8.0 program set. The conductance value thus obtained was 53.0 ± 7.2 pS.

**Reagents and Statistics**—All chemicals were of the highest purity commercially available. Reported results are typical of at least three replicates for each condition, and error bars refer to the S.E. p values were calculated with Student’s t test.

**RESULTS AND DISCUSSION**

*Drosophila S2R*+ cells were permeabilized with digitonin and energized with succinate in the presence of ADP and P; an ATP-hydrolyzing system was present to maintain constant [ADP] and synchronize F-ATPases in the direction of ATP synthesis. A train of Ca2+ pulses was then added to determine the CRC, i.e. the threshold matrix Ca2+ load necessary to open the mCRC (1) (Fig. 1A, trace a). Next, we incubated permeabilized S2R+ cells in the absence of respiratory substrates, and then energized mitochondria with ATP in the presence of an ATP-regenerating system to maintain constant [ATP] and synchronize F-ATPases in the direction of ATP hydrolysis (Fig. 1A, trace b). The CRC was much larger in ATP-hydrolyzing than in ATP-synthesizing mitochondria (Fig. 1, compare traces a and b). Due to formation of glucose-6-P, the concentration of P; decreases in the ATP synthesis experiments, and conversely, due to hydrolysis of P-creatine, it increases in the ATP-hydrolyzing protocols. However, in the presence of oligomycin, the CRC was only slightly higher than that observed during ATP synthesis (Fig. 1, trace a’), and in the presence of 5 mM P; under conditions of ATP hydrolysis, the CRC was indistinguishable from that observed at 1 mM P; (Fig. 1, trace b’). Thus, the difference between ATP-synthesizing and ATP-hydrolyzing mitochondria cannot be explained by differences in the P; concentration. Note that the difference in CRC was also not due to different levels of the membrane potential, which was the same in ATP-synthesizing (Fig. 1B, trace a) and ATP-hydrolyzing mitochondria (Fig. 1B, trace b), and responded appropriately to oligomycin and uncoupler.

The threshold Ca2+ was increased 3-fold in ATP-hydrolyzing relative to ATP-synthesizing mitochondria (Fig. 1C), and the effect was not due to the different nucleotide as such because ADP and ATP displayed indistinguishable effects on the CRC when F-ATPase catalysis was blocked with oligomycin in the presence of Mg2+, and mitochondria were energized with succinate (Fig. 1D). We could also exclude participation of the endoplasmic reticulum because Ca2+ uptake was not observed in the presence of ATP plus oligomycin (results not shown; see Ref. 6). The intriguing effect of enzyme catalysis described in Fig. 1A is also observed for the mammalian PTP (6) and represents a first indication that the mCRC may originate from the F-ATPase as well. This effect could be due to the different conformations of F-ATPase during ATP synthesis and hydrolysis (see e.g. Ref. 19) and suggests that the sense of rotation of F-ATPase influences the mCRC of *Drosophila* mitochondria, possibly by modulating accessibility of Ca2+-sensitive site(s) that trigger channel opening (20).

The well characterized F-ATPase inhibitor Bz-423 binds the OSCP subunit of the enzyme in mammalian mitochondria (21) and inhibits both ATP synthesis and hydrolysis (22) while sensitizing the PTP to opening (6). Interestingly, the Ca2+ load necessary to trigger mCRC opening was decreased by Bz-423 in a concentration-dependent manner (Fig. 1E).

CyPD, a key regulator of the mammalian PTP, also binds the OSCP subunit of the F-ATPase, presumably at the same site as Bz-423, because the latter displaces CyPD from the enzyme complex (6). CyPD binding can be selectively inhibited by cyclosporin A (CsA) or by genetic ablation of CyPD, and both manipulations remarkably desensitize the PTP to Ca2+ in that its opening requires about twice the Ca2+ load necessary to open the PTP in untreated mitochondria (23). The *Drosophila* genome encodes 14 different CyPs (24). *D. melanogaster* CyP1 has an N-terminal sequence that according to Mitoprot (25) confers high probability of mitochondrial import, yet full sequence analysis led to the conclusion that no mitochondrial CyP is present in this species (24). Consistent with this conclusion, we could not detect mitochondrial localization of a CyP1-GFP fusion protein in S2R+ cells (results not shown).

We prepared a cDNA construct with an N-terminal *Drosophila* mitochondrial targeting sequence followed by the human CyPD coding sequence and by a C-terminal HA tag. Transfected cells expressed the human CyPD construct, which was recognized by both the CyPD and the HA antibodies, with the increase of molecular weight expected of the HA tag (Fig. 2A, top left panel). The protein largely localized to mitochondria as judged (i) by Western blot analysis of cytosolic and mitochondrial fractions after subcellular fractionation (Fig. 2A, bottom left panel); and (ii) by colocalization with MitoTracker Red CMXRos in fixed cells immunostained with a mouse anti-HA antibody followed by a secondary fluoresceinated antibody against mouse IgG (Fig. 2A, right panel).

Analysis of the CRC in cells co-transfected with the expression vector pActCyPD-HA and the pCoPuro selection vector as compared with cells containing only the selection vector
showed that CyPD expression reduces the threshold Ca\(^{2+}\) load required to induce Ca\(^{2+}\) release (Fig. 2B, compare traces a and b). The effect of CyPD could not be counteracted by CsA (Fig. 2B, trace c), however, irrespective of the concentration of P\(_i\) (Fig. 2C), which in mammalian mitochondria favors CyPD binding to the F-ATPase, resulting in pore sensitization to Ca\(^{2+}\) (26, 27). The reason why CsA does not inhibit the mCrC after enforced expression of CyPD remains unclear, but we note (i) that D. melanogaster OSCP has a larger number of negative charges in the putative CyPD binding region, which could increase binding affinity; and (ii) that PTP inhibition does not depend on inhibition of CyPD enzymatic activity (28).

At variance from the PTP, the *Drosophila* mCrC is impermeable to sucrose (1). Of note, the size of the *Drosophila* mCrC was unaffected by expression of human CyPD because no Ca\(^{2+}\)-induced swelling could be observed after the addition of enough Ca\(^{2+}\) to induce mCrC opening (not shown) irrespective of whether naive (Fig. 2D, trace a) or CyPD-expressing permeabilized cells (Fig. 2D, trace b) were used. S\(_2\)R\(^{+}\) cells were derived from late embryonic stages (12). To assess whether the low exclusion size and inhibitory response to P\(_i\) of the mCrC are restricted to early developmental stages, or rather are defining properties of the *Drosophila* channel, we also measured the swelling response in mitochondria isolated from *white* \(^{1118}\) 3rd instar larvae or adult flies. Neither mitochondrial preparation underwent Ca\(^{2+}\)-induced swelling, which could instead be readily induced by the addition of the pore-forming peptide alamethicin (Fig. 2D, traces c and d).

In the absence of rotenone, succinate induces reverse electron flow through complex I and leads to generation of reactive oxygen species (29). Rotenone is a potent inhibitor of the mammalian PTP when succinate is used as the substrate (30), and inhibition of reverse electron flow at complex I provides a plausible mechanism for this inhibition because reactive oxygen species increase the probability of pore opening through thiol oxidation (31, 32). Remarkably, rotenone increased nearly 4-fold the CRC of *Drosophila* mitochondria from both larvae and adults (Fig. 2E). Thus, lack of swelling after mCrC opening...
The dimer preparation was eluted from the gel and incorporated into asolectin planar bilayers for determination of channel activity (6). The addition of Ca\(^{2+}\), Bz-423, phenylarsine oxide, and copper-o-phenanthroline induced opening of a channel with a prevalent single channel conductance of 53 pS (Fig. 3, B and C), which was consistently observed in different dimer preparations. The addition of \(\gamma\)-imino ATP and Mg\(^{2+}\) induced channel closure within a few seconds, as also demonstrated by the amplitude histograms obtained from current traces before and after the addition of the modulators (Fig. 3C). Further electrophysiological experiments confirmed that Drosophila F-ATPase dimers allow the passage of Ca\(^{2+}\) currents (results not shown). Thus, under conditions of oxidative stress and in the presence of Ca\(^{2+}\) and Bz-423, the F-ATPase of Drosophila forms channels whose conductance is 10-fold smaller than that of the mammalian PTP (6) and 6-fold smaller than that of yeast (9). These features are a perfect match to those defined for the mCrC in permeabilized S\(_{2R}\) cells (1) and leave little doubt that the mCrC is the PTP of D. melanogaster. Thus,
The N-terminal and fungi c-rings of 10–15 subunits have been observed (40). The c-ring has 8 c-subunits, whereas in prokaryotes, chloroplasts and mitochondria of mammals, the c-ring is formed by identical c-subunits arranged to form a barrel, the actual conductance channels both in the native membrane and during electrophysiological experiments, where dimers could in principle form a channel with a conductance of 500 pS in mammals and of a mere 53 pS in Drosophila, and of 300 pS in yeast where the c-ring has 10 subunits (40). Our findings are therefore not consistent with the idea that PTP channel forms within the c-ring (39), whose actual conductance and Ca²⁺ dependence remain controversial (44–46); see also Refs. 47–49 for discussion.

Given that we readily observe channel formation with F-ATPase dimers but not monomers (6, 9), and that dimerization-resistant yeast strains display resistance to PTP opening (9), our working hypothesis is that the pathway for solute permeation forms between dimers (or higher order structures) (20). Intriguingly, cryoelectron tomography established that F-ATPase dimers from Drosophila flight muscle have a unique supramolecular structure, with two parallel rows at the high-curvature edge of cristae vesicles (43). It is possible that this supramolecular assembly prevents formation of high-conductance channels both in the native membrane and during electrophysiological experiments, where dimers could in principle be organized in higher order structures.

Depending on the open time, the mammalian PTP could be involved both in apoptosis induction and in Ca²⁺ homeostasis (50). Prolonged openings cause mitochondrial depolarization, osmotic swelling, outer mitochondrial membrane rupture, and release of apoptogenic proteins such as cytochrome c; transient openings, on the other hand, may be involved in physiological

![Image](https://example.com/image.png)
Ca\(^{2+}\) homeostasis and may protect mitochondria from Ca\(^{2+}\) overload (10, 11), as indicated by studies in isolated cardiomyocytes, CyPD knock-out mice, adult cortical neurons, and spinal chord mitochondria (51–54). It is tempting to speculate that the different PTP conductances observed in mammals, \textit{Drosophila}, and yeast underscore different physiological functions. In particular, due to its smaller size, the \textit{Drosophila} PTP could be involved in Ca\(^{2+}\) homeostasis rather than in apoptosis (1, 11), consistent with the finding that the mitochondrial pathway is not essential in most cases of \textit{Drosophila} apoptosis (55–59). The discovery that the \textit{Drosophila} PTP forms from F-ATPase will allow testing of this hypothesis with the powerful methods of \textit{Drosophila} genetics.

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