Mitochondrial permeability transition in the crustacean *Artemia franciscana*: absence of a calcium-regulated pore in the face of profound calcium storage

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Mitochondrial permeability transition in the crustacean *Artemia franciscana*: absence of a calcium-regulated pore in the face of profound calcium storage. *Am J Physiol Regul Integr Comp Physiol* 289: R68–R76, 2005. First published February 17, 2005; doi:10.1152/ajpregu.00844.2004.—When mammalian mitochondria are exposed to high calcium and phosphate, a massive swelling, uncoupling of respiration, and release of cytochrome *c* occur. These changes are mediated by opening of the mitochondrial permeability transition pore (MPTP). Activation of the MPTP in vivo in response to hypoxic and oxidative stress leads to necrotic and apoptotic cell death. Considering that embryos of the brine shrimp *Artemia franciscana* tolerate anoxia for years, we investigated the MPTP in this crustacean to reveal whether pore opening occurs. Minimum molecular constituents of the regulated MPTP in mammals are believed to be the voltage-dependent anion channel, the adenine nucleotide translocators, and cyclophilin D. Western blot analysis revealed that mitochondria from *A. franciscana* possess all three required components. When measured with a calcium-sensitive fluorescent probe, rat liver mitochondria are shown to release matrix calcium after addition of ≥100 μM extramitochondrial calcium (MPTP opening), whereas brine shrimp mitochondria continue to take up extramitochondrial calcium and do not release internal stores even up to 1.0 mM exogenously added calcium (no MPTP opening). Furthermore, no swelling of *A. franciscana* mitochondria in response to added calcium was observed, and no release of cytochrome *c* could be detected. HgCl₂-dependent swelling and cytochrome *c* release were readily confirmed, which is consistent with the presence of an “unregulated pore.” Although the absence of a regulated MPTP in *A. franciscana* mitochondria could contribute to the extreme hypoxia tolerance in this species, we speculate that absence of the regulated MPTP may be a general feature of invertebrates.

Mitochondria; mitochondrial permeability transition pore; anoxia; apoptosis

MITOCHONDRIA ARE CONTRIBUTORS to both the energetic processes necessary for life and to signaling events that lead to death of eukaryotic cells (57). Under many physiological states, mitochondria serve their well-known role in coupling respiration to ATP synthesis (56). Under certain stressful conditions, this function can dramatically change, and mitochondria can assume a central role in the pathways leading to necrotic and apoptotic cell death (8, 35, 54, 57). When mammalian mitochondria are exposed to high calcium concentrations in the presence of the coactivator Pi, especially if accompanied by oxidative stress and adenine nucleotide depletion, a large swelling, uncoupling of respiration, and release of cytochrome *c* can be observed (30, 35, 39, 73). These phenomena are due to a sudden increase in permeability of the inner mitochondrial membrane to solutes with a molecular mass of up to ~1,500 Da, a phenomenon known as the mammalian mitochondrial permeability transition (7, 44). Embryos of the brine shrimp *Artemia franciscana* are exceptional in their ability to tolerate anoxia at room temperature for years (15) and to maintain viability under conditions that are known to open the mitochondrial permeability transition pore (MPTP) in mammalian species, thereby leading to cytochrome *c* release and cell death (33, 55, 73). In the present study, we therefore investigated the status of the MPTP in *A. franciscana* with the hypothesis that the high tolerance to anoxia in this species could be due in part to nonfunctional MPTP, i.e., one that is resistant to calcium-induced release of cytochrome *c*.

Mitochondrial permeability transition is mediated by a multiprotein complex, which can be defined as a voltage-dependent, cyclosporin A-sensitive, and calcium-activated inner membrane channel (5). The minimum constituents of the regulated MPTP are believed to be the voltage-dependent anion channel (VDAC), the adenine nucleotide (ATP/ADP) translocators (ANTs), and the peptidyl-prolyl cis-trans isomerase (PPI) cyclophilin D (17, 32, 64). However, recent evidence has shown that the MPTP can form in the absence of ANT (49), and alternative models have been proposed (40). Several inducers and inhibitors of the mammalian mitochondrial permeability transition have been described that interact with different components of the MPTP and are useful for its characterization. Atractyloside, calcium, and oxidative stress increase the probability of pore opening, whereas bongkrekic acid, low pH, magnesium, and the adenine nucleotides ATP and ADP decrease the pore opening probability by interacting with ANT (32, 36). The immunosuppressant peptide cyclosporin A acts as a potent inhibitor of mammalian MPTP opening by preventing cyclophilin D binding to ANT, whereas sanglifehrin A blocks pore opening by inhibiting the PPI activity of cyclophilin D (14, 33, 34, 71). A new class of drugs, such as Ro68–3400 (12), inhibits mammalian mitochondrial permeability transition by binding to VDAC-1. Furthermore, thiol-reactive reagents such as the mercaptide forming reagent HgCl₂ (40, 72) or phenylarsine oxide, which specifically cross-links vicinal sulphydryl groups (69), and the peptide mastoparan (63) are well known as inducers of the mammalian mitochondrial permeability transition.

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Despite an enormous amount of literature about the mammalian MPTP (5, 7, 17, 31, 74), little is known about MPTPs in nonmammalian species. In vitro studies have shown that in lipid vesicles reconstituted ANTs from Neurospora crassa can form mega-channels that exhibit characteristics similar to the MPTP (response to calcium, ADP, and bongkrekic acid) (11). However, the occurrence of an in vivo MPTP in N. crassa mitochondria was not examined. Mitochondria from the yeast species Saccharomyces cerevisiae exhibit a permeability transition pore that is inhibited by ADP and exhibit comparable size-exclusion properties to the homologues structure in mammalian mitochondria, but these mitochondria are not induced by calcium and are cyclosporin A insensitive (47). In addition, no calcium-induced transition could be found in mitochondria from the yeast Endomyces magnusii (18). Investigations of purified potato and wheat mitochondria show a calcium-sensitive permeability transition that is inhibited by cyclosporin A in the presence of dithiothreitol (3, 70). Isolated liver mitochondria from the great green goby (Zosterisessor ophiophallus) show calcium-induced swelling if incubated in the presence of Pi, but the amount of calcium required to induce mitochondrial permeability transition was substantially higher than that needed in mitochondria from rat liver (67).

As mentioned above, embryos of A. franciscana are perhaps the quintessential example of an anoxia-tolerant invertebrate. In response to oxygen limitation, these embryos enter metabolic and developmental quiescence, during which the rate of energy flow approaches an ametabolic state (15, 37). Many mitochondrial functions are downregulated during anoxia, including protein synthesis (50, 51), transcription (22, 23), mRNA turnover (21), and uncoupled proton flux (25). Similar cellular processes are severely depressed in the nuclear-cytoplasmic compartment as well (1, 16, 43, 68), and there is a high correlation between the degree of metabolic depression and the length of anoxia tolerance in aquatic animals (38).

Although uncoupled proton flux is reduced across the inner membrane under severe hypoxia in A. franciscana (25), it is nevertheless inconceivable that the membrane potential is not dissipated eventually during prolonged anoxia. Dissipation of the membrane potential promotes the opening of the MPTP, particularly in concert with the elevation of intracellular calcium (6, 61, 62). A large increase in intracellular calcium is a hallmark change observed in many cell types exposed to prolonged anoxia (42). Thus an open question exists as to how A. franciscana avoids apoptotic cell death during long-term anoxia, an environmental challenge that should foster cytochrome c release from mitochondria. In this study, we sought answers to the following specific questions. Do mitochondria of A. franciscana embryos possess the macromolecular components of the MPTP? Do isolated mitochondria from these embryos have the capacity to accumulate calcium? If so, is opening of the MPTP regulated by the extramitochondrial calcium level and is cytochrome c released during the open state? If not, can mitochondrial swelling (indicative of pore opening) and cytochrome c release be promoted by less specific/nonphysiological agents like HgCl2? What are the size-exclusion properties of such a pore when opened? Answers to these questions should improve not only our understanding of mechanisms for anoxia tolerance in A. franciscana embryos but also provide insights into the distribution and evolution of regulated and nonregulated MPTPs.

### MATERIALS AND METHODS

**Chemicals.** Chemicals for mitochondria isolation and incubation, including potassium phosphate monobasic, potassium chloride, HEPES, and Tris, were obtained from Fisher Scientific (Fair Lawn, NY). The MPTP inhibitor cyclosporin A, the activators mastoparan and atracylcoside, and horse heart cytochrome c were purchased from Sigma-Aldrich (St. Louis, MO). Sucrose came from Ferro Pfanstiehl Laboratories (Waukegan, IL). The calcium probe fluo-5N was obtained from Molecular Probes (Eugene, OR). All other reagents were of analytical grade and from Sigma-Aldrich. Water for solution preparation was purified with a Milli-Q reagent water system (Millipore, Billerica, MA).

**Isolation of mitochondria.** Rat liver mitochondria were isolated from male Sprague-Dawley rats (250–300 g). Rats were euthanized by CO2 asphyxiation, and the livers were quickly removed, chopped into small cubes, and homogenized in ice-cold 0.25 M sucrose, 1 mM EGTA, and 20 mM K+-HEPES (pH 7.2) using a motor-driven glass-Teflon homogenizer at 300–600 rpm for five or six passages (Thomas Scientific, Swedesboro, NJ). The homogenate was centrifuged for 15 min at 600 g and 4°C, and the supernatant was removed and centrifuged at 9,750 g for 15 min to pellet the mitochondrial fraction. The resulting pellet was gently resuspended and centrifuged two more times using 0.25 M sucrose, 0.025 mM EGTA, and 20 mM K+-HEPES (pH 7.2). All experiments were conducted in accordance with the Institute for Animal Research Guide for the Care and Use of Laboratory Animals (protocol approved by the Institutional Animal Care and Use Committee, protocol no. 02-023).

Dehydrated, encysted gastrulae of A. franciscana were obtained from Sanders Brine Shrimp and stored at −20°C until use. Embryos were hydrated in 0.25 M NaCl at 0°C for at least 4 h before incubation. Hydrated cysts (15 g) were then incubated at room temperature in 500 ml of 0.25 M NaCl on a rotary shaker at 1,100 rpm for 8 h (52). After this developmental incubation, the embryos were dechorionated in antiformin solution (1% hypochlorite from bleach, 60 mM NaCO3, and 0.4 M NaOH) followed by a rinse in 1% thiosulfate and multiple washings in ice-cold 0.25 M NaCl as previously described (52). After the embryos were filtered and blotted dry, 10 g were homogenized in ice-cold isolation buffer 1 (0.5 M sucrose, 150 mM KCl, 1 mM EGTA, 0.5% (wt/vol) fatty acid-free BSA, and 20 mM K+-HEPES, pH 7.5) using a glass-Teflon homogenizer at 1,000–1,100 rpm for six passages. The homogenate was centrifuged for 10 min at 1,000 g and 4°C, and the supernatant was removed and centrifuged at 9,000 g for 15 min to pellet the mitochondrial fraction. The resulting pellet was gently resuspended in ice-cold isolation buffer 2 (0.5 M sucrose, 150 mM KCl, 0.025 mM EGTA, 0.5% (wt/vol) fatty acid-free BSA, and 20 mM K+-HEPES, pH 7.5) and centrifuged again at 9,000 g for 15 min. The final pellet was resuspended in ~1 ml of isolation buffer 2 and contained ~20 mg protein/ml. Mitochondrial samples that were used in the SDS-PAGE and Western blot analyses were prepared the same way, except that a BSA-free isolation buffer 2 was used. The composition and resulting isosmotic pressure (920 mosM) of isolation media for A. franciscana mitochondria are based on studies of the intracellular osmolyte system of these embryos (24), as well as on the optimal functional character of the mitochondria for oxidative phosphorylation (25, 53) and other processes (21–23, 50–52).

To obtain mitochondria from nauplius larvae of A. franciscana, encysted gastrulae were allowed to develop for 24 h at room temperature in 0.3% (wt/vol) instant ocean (Aquarium Systems, Monitor, OR) on a rotary shaker at 1,100 rpm. We separated hatched nauplii from unhatched cyst and cyst shells using a separatory funnel, and mitochondria were isolated as described for hydrated cysts but without the dechorionation procedures. Mitochondrial protein was quantified using a modified Lowry assay according to Peterson (60) with BSA as the standard.
Fluorescence-based measurements of calcium. Calcium-induced fluorescence was measured in 96-well plates in a fluorescence plate reader (Victor 3 multilabel counter, Perkin-Elmer, Wellesley, MA). Typically, assays of mitochondria (0.5 mg/ml) from A. franciscana were carried out at 25°C in 500 mM sucrose, 150 mM KCl, 1 mM KH2PO4, 5 μM rotenone, and 20 mM K+HEPES, pH 7.5. Rat liver mitochondria (0.5 mg/ml) were assayed at 25°C in 200 mM sucrose, 1 mM KH2PO4, 5 μM rotenone, 20 mM HEPES, and 10 mM Tris, pH 7.2. Alternatively, calcium uptake was measured in the buffer systems indicated in the legend to Fig. 4. Studies with energized mitochondria were performed in the presence of 5 mM succinate. To detect calcium, the calcium-sensitive fluorescence probe fluo-5N was added at a final concentration of 1 μM (9), and fluorescence was excited from above the wells 20 min after addition of calcium at concentrations specified. We measured the green fluorescence of fluo-5N using an excitation filter of 485 nm and an emission filter of 535 nm. Fluorescence was expressed as the percentage of the maximal fluorescence (%Fmax) obtained when the calcium probe was completely saturated with calcium in the respective buffer system. To obtain Fmax, the fluorescence was measured at intervals across a range of 1–1,000 μM calcium. Data were fitted with the computer program SigmaPlot 2001 for Windows version 7.101 (SPSS, Chicago, IL) to the function: F = (K × X + Fmax)/(1 + K × X), where F is the measured fluorescence, X the calcium concentration, Fmax is the maximal obtainable fluorescence, and K is the stoichiometric binding constant (1/Kd) of calcium to fluo-5N. The dissociation constant (Kd) estimated was ~100 μM and within the range stated by the supplier.

Mitochondrial volume changes and determination of released proteins. Mitochondrial volume changes were measured from the absorbance changes at wavelength (λ) = 540 nm (62) using a dual-beam spectrophotometer (Cary-100, Varian, Walnut Creek, Ca), equipped with magnetic stirring and thermostatic control. With this method, a decrease in absorbance indicates an increase in mitochondrial volume. Measurements of rat mitochondria (0.5 mg protein/ml) were carried out at 25°C in 100 mM succrose, 100 mM KCl, 1 mM KH2PO4, 5 μM rotenone, 25 μM EGTA, and 20 mM K+HEPES, pH 7.4. Mitochondria (0.5 mg protein/ml) from A. franciscana were incubated at 25°C in 500 mM succrose, 150 mM KCl, 1 mM KH2PO4, 5 μM rotenone, 25 μM EGTA, and 20 mM K+HEPES, pH 7.4. Mitochondria without PEGs was monitored in 200 mM KCl and 20 mM K+HEPES, pH 7.5. Alternative buffer systems were used as indicated in the legend to Fig. 4. Studies with energized mitochondria were performed in the presence of 5 mM succinate. calcium was added in concentrations specified.

To measure the release of mitochondrial proteins, samples (1 mg protein/ml) were incubated in the respective buffer for 15 or 30 min in microtubes and then centrifuged at 10,000 g for 10 min at 4°C to pellet the mitochondria. Four milliliters of the supernatants containing the released proteins were transferred into Centricon YM-3 centrifugal filter devices (Millipore, Bedford, MA) with a molecular size exclusion of 3,000 Da. The supernatants were concentrated to ~100 μl by centrifugation at 4°C and stored at ~80°C until analyzed. We quantified released protein using a Coomassie Plus protein assay kit (Pierce, Rockford, IL) and SDS-PAGE and Western blot analyses (see SDS-PAGE and Western blot analyses).

Measurement of size-exclusion limits. The solute size-exclusion method was used to estimate the exclusion limit of the mercury-induced permeability transition pore in A. franciscana mitochondria. Initial studies showed that mercury-induced swelling was strongly inhibited in sucrose-containing medium, which indicated a size-exclusion limit of ~350 Da. Inhibition of mercury-induced swelling was monitored more thoroughly by additions of 100 mosM polyethylene glycols (PEGs) of varying molecular weights, as indicated in Fig. 7. Absorbance was recorded after any significant volume changes had ceased, and the values were expressed as the percentage of swelling in PEG-free medium. However, in measurements with 1,000 molecular weight PEG, the time needed to reach a new steady-state volume was significantly longer than in measurements with PEGs of lower molecular weight. Varying the molecular weight of PEGs reveals a size-exclusion property because no inhibition of swelling is observed if the PEGs are able to penetrate the mitochondria (63). Because PEGs are nonideal solutes with respect to their osmotic characteristics, the concentration required to produce a particular osmotic pressure is a function of the PEG molecular weight (63). Stock solutions of PEGs were made up at 500 mM, and the osmotic pressure of solutions was measured with a microosmmeter (Osmette 5004, Precision, Natick, MA). The amount of stock solution to yield 100 mosM was calculated and added accordingly. Control swelling without PEGs was monitored in 200 mM KCl and 20 mM K+HEPES, pH 7.5. The average osmolarity of solutions (150 mM KCl and 20 mM K+HEPES, pH 7.5) containing PEGs of different molecular weights was 399 ± 20 (SD) mosM (n = 5).

**SDS-PAGE and Western blot analyses.** Samples containing mitochondrial proteins from A. franciscana embryos were subjected to 15% SDS-PAGE using a Bio-Rad Mini-PROTEAN 3 Cell (Bio-Rad Laboratories, Hercules, CA). Samples analyzed for cytochrome c were loaded on a discontinuous polyacrylamide gel (4% stacking gel and then 10 and 15% separation gels). Proteins were denatured at 95°C for 5 min in 4× sample buffer (2% SDS, 25% glycerol, 5% β-mercaptoethanol, 0.01% bromphenol blue, and 62.5 mM Tris-HCl, pH 6.8), and 15–20 μg of protein were loaded per lane. After electrophoresis, proteins in the gel were visualized by silver staining (Silver Stain plus kit, Bio-Rad Laboratories) or electrophoretically transferred onto a nitrocellulose membrane (0.2 μm, Bio-Rad) in a transfer buffer (192 mM glycine, 20% methanol, 0.025% SDS, and 25 mM Tris) by use of a Bio-Rad Mini Trans-Blot. Membranes were stained with Ponceau S (Sigma-Aldrich) to confirm transfer of the proteins. Blocking of membranes was performed using casein (Vector Laboratories, Burlingame, CA) for 30 min. Rabbit anti-cyclophilin D polyclonal antibody was a generous gift from James D. Lechleiter (University of Texas Health Science Center, San Antonio, TX). Goat anti-ANT (n-19), goat anti-VDAC-1 (n-18) (Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-cyclophilin D were used as primary antibodies at 1:2,000 dilution, whereas goat anti-cytochrome c (C-20) polyclonal antibody (Santa Cruz Biotechnology) was diluted 1:500. The blots were incubated overnight with the primary antibody in casein solution at 4°C. Biotinylated goat anti-rabbit IgG and rabbit anti-goat IgG (Vector Laboratories) were used as secondary antibodies at dilutions of 1:1,000. We stained blots using Vectastain ABC-AMP kit (Vector Laboratories), following the protocol of the manufacturer. Briefly, membranes were washed with casein solution in the presence of avidin and biotin and then probed for 30 min at room temperature with the respective secondary antibody. The blots were washed with 0.1 M Tris (pH 9.5) and developed by use of DuoLux substrate for alkaline phosphatase (Vector Laboratories). Fluorescence or chemiluminescence was visualized with a Typhoon 8600 (Perkin-Elmer) imager.

**Statistical analyses.** The statistical significance of data sets was evaluated by performing t-tests, P ≤ 0.05, with SigmaPlot 2001 for Windows version 7.101 (SPSS).

**RESULTS**

Activation of the MPTP in vivo, in response to hypoxic and oxidative stress, leads to necrotic and apoptotic cell death (8, 31, 54). Considering that embryos of A. franciscana tolerate anoxia for years (15), we investigated the MPTP in this crustacean to reveal whether pore opening occurs. Western blot analyses demonstrated that mitochondria isolated from A. franciscana contain all components of the regulated mitochondrial transition pore (Fig. 1). VDAC (Fig. 1c), molecular mass of ~32 kDa (12); ANT (Fig. 1d), molecular mass of ~30 kDa (10); and PPI cyclophilin D (Fig. 1f), molecular mass of ~18 kDa (28) could be detected with polyclonal antibodies raised against these proteins. Although nonspecific interactions be-
between the anti-cyclophilin D polyclonal antibody and several mitochondrial proteins were found (Fig. 1f), cyclophilin D could be identified by its molecular mass of ~18 kDa (28). Negative controls (Fig. 1, b and e) were included to avoid false positives that could result from direct binding of secondary antibodies to the proteins.

**Effect of exogenous calcium on mitochondria from A. franciscana** from A. franciscana and rat liver. Energized and deenergized mitochondria from A. franciscana and rat liver were incubated in presence of the calcium probe fluo-5N at exogenously added calcium concentrations between 0 and 1 mM. Because the calcium probe fluo-5N cannot penetrate the mitochondrion, the measured values expressed in %F_max correlate to the external free calcium concentration for values below the saturation signal of the calcium probe.

Calcium uptake by energized mitochondria (0.5 mg protein/ml) was found for both species. As expected, the deenergized mitochondria from both species did not have any capacity for calcium uptake. If no calcium was added, the %F_max value for energized mitochondria was significantly lower than that for deenergized mitochondria or the control (mitochondrial-free) medium (t-test, n = 3, P < 0.05; Fig. 2). In other words, energized mitochondria of both species were able to lower the free calcium concentration below the background calcium contamination introduced from glassware. However, mitochondria from the two species differed dramatically in their responses to exogenously added calcium. After addition of 0.1 mM calcium to energized rat mitochondria (Fig. 2B), a large increase in fluorescence was observed compared with that shown at the 0 mM calcium value, whereas no increase was found for energized mitochondria of A. franciscana (Fig. 2A). Furthermore, the increase in %F_max found for rat mitochondria after addition of 0.1 mM calcium was significantly higher in samples containing either energized or deenergized mitochondria compared with control assay buffer, indicating that an extramitochondrial calcium threshold was reached, causing the opening of the MPTP and release of calcium from intramitochondrial stores (t-test, n = 3, P < 0.05; Fig. 2B). Only small changes in fluorescence are seen for rat mitochondria as calcium is raised further from 0.1 to 1.0 mM. Note that the fluorescence response to free calcium is nonlinear, as judged most easily by the pattern in control samples.

In contrast to the results for rat liver mitochondria, energized mitochondria from A. franciscana (Fig. 2A) are able to lower the external calcium concentration, compared with controls, across the entire range of experimental calcium. From 0 to ~0.5 mM exogenous calcium, there is no statistical change in the extramitochondrial level of free calcium that is maintained by energized mitochondria. Above 0.5 mM, the free calcium levels begin to move gradually upward but, importantly, remain well below control values even after addition of 1 mM calcium (Fig. 2A). Thus the capacity for calcium uptake by mitochondria from A. franciscana is dramatically greater than...
that seen for rat liver mitochondria. Second, although a clear calcium-dependent opening of the MPTP occurs at ~0.1 mM calcium in rat liver mitochondrial, no calcium-dependent MPTP opening is observed for A. franciscana mitochondria because the calcium level in the presence of energized mitochondria never reaches, much less ever exceeds, the control values.

Differential effects of calcium on mitochondria from rat liver vs. A. franciscana were also revealed by monitoring mitochondrial volume changes in response to calcium. Energized rat mitochondria swelled rapidly in sucrose-based medium after addition of 0.1 mM calcium (Fig. 3A), as seen by the decline in absorbance at 540 nm, again indicative of MPTP opening. However, if energized mitochondria from A. franciscana were treated with 1 mM calcium, an apparent decrease in mitochondrial volume (increase in absorbance) was observed (Fig. 3A). This increase in absorbance is dependent on mitochondrial calcium uptake; deenergized mitochondria did not respond to an addition of calcium (Fig. 3B). Thus, by this independent measure of swelling, a calcium-regulated opening of the MPTP was not detectable in A. franciscana mitochondria.

It is appropriate to emphasize that isolated mitochondria were evaluated under isosmotic conditions for the respective species (~300 mosM for rat mitochondria and ~920 mosM for mitochondria from A. franciscana). However, to exclude any possibility that lack of MPTP opening in A. franciscana was due to osmotic differences among buffers, these mitochondria were challenged with 1 mM calcium under hypsomotic conditions in the incubation buffers used for rat mitochondria. Because of hypsomotic stress, a slower increase in absorbance after addition of calcium was observed (Fig. 4, A and B) compared with that seen in isosmotic buffer (Fig. 3A). Nevertheless, a decrease in absorbance (i.e., swelling) was not measurable, verifying that the absence of calcium-induced MPTP opening is apparently a species-specific difference and not a consequence of buffer composition. Similarly, the capacity for calcium uptake by A. franciscana mitochondria was assessed at 0.2 mM calcium in media used for rat liver mitochondria. Although physiologically compromised in this buffer, the energized shrimp mitochondria still showed significant calcium uptake, and a net release of calcium from the mitochondrial matrix was not seen (Fig. 4, inset), again consistent with the absence of MPTP opening.

Response of A. franciscana mitochondria to MPTP inhibitors and inducers. In addition to the classical MPTP inducer pair (i.e., calcium and phosphate), the effects on mitochondrial volume of the nonphysiological inducers mastoparan, atracyloside, phenylarsine oxide, and mercuric chloride and the inhibitor cyclosporin A were investigated. No differences in absorbance in response to Ca\(^{2+}\) can be observed (i.e., no MPTP opening) in any buffer system investigated, and energized mitochondria are able to reduce Ca\(^{2+}\) below the amount added in all buffers tested (inset). Single representative traces of absorbance are shown, but at least 3 independent measurements were conducted and results were reproducible. Each bar (in inset) represents the mean ± SD of n = 4–6 different measurements. *Statistically different from control (P < 0.05).

Fig. 3. Effects of Ca\(^{2+}\) in the presence of 1 mM phosphate on isolated mitochondria from 8-h cysts of A. franciscana and rat liver. Mitochondrial volume changes after addition of 1 mM Ca\(^{2+}\) to mitochondria isolated from 8-h cysts of A. franciscana (0.5 mg protein/ml in 500 mM sucrose, 150 mM KCl, 1 mM KH\(_2\)PO\(_4\), 5 μM rotenone, 25 μM EGTA, and 20 mM K-HEPES, pH 7.5) and after addition of 0.1 mM calcium to energized rat mitochondria (trace C) (0.5 mg of protein/ml in 100 mM sucrose, 100 mM KCl, 1 mM KH\(_2\)PO\(_4\), 5 μM rotenone, 25 μM EGTA, and 20 mM K-HEPES, pH 7.4) are shown. Abs, absorbance.

Fig. 4. Influence of buffer composition on the Ca\(^{2+}\) responses of mitochondria from A. franciscana (8-h cysts). Addition of 1 mM Ca\(^{2+}\) to mitochondria incubated under hypsomotic conditions in buffer used for rat studies (trace A) on 100 mM sucrose, 100 mM KCl, 20 mM K-HEPES, pH 7.4 and 320 mosM) leads to a slow increase in absorbance measured at 540 nm. Reducing the osmolarity to 250 mosM (trace B: 200 mM sucrose, 20 mM HEPEs, 10 mM Tris, pH 7.2) leads to an even slower increase in absorbance after Ca\(^{2+}\) addition. These patterns correlate with the Ca\(^{2+}\) uptake measured after addition of 0.2 mM Ca\(^{2+}\) to the respective media (inset). Importantly, no decrease in absorbance in response to Ca\(^{2+}\) can be observed (i.e., no MPTP opening) in any buffer system investigated, and energized mitochondria are able to reduce Ca\(^{2+}\) below the amount added in all buffers tested (inset). Single representative traces of absorbance are shown, but at least 3 independent measurements were conducted and results were reproducible. Each bar (in inset) represents the mean ± SD of n = 4–6 different measurements. *Statistically different from control (P < 0.05).
sporin A insensitive (Fig. 6F), suggesting an unregulated mitochondrial permeability transition (40). Other inducer combinations that are known to be effective on the regulated MPTP (36, 63, 74) elicited no change in volume (Fig. 6, A, B, and D) of mitochondria from *A. franciscana*, again confirming the absence of a regulated MPTP. If the experiments were repeated under energized conditions (addition of 5 mM succinate and 5 

μM rotenone), similar traces were obtained (data not shown). It is appropriate to note that, after addition of 20 μM HgCl₂, energized mitochondria did not respond to addition of calcium (data not shown); this lack of response is in contrast to the apparent decrease in mitochondrial volume elicited by calcium above (Fig. 3A). Thus pretreatment with HgCl₂ apparently promotes a dissipation of the mitochondrial membrane potential, thereby preventing calcium uptake.

Size-exclusion limit of the unregulated MPTP and inhibition of swelling by sucrose. To further characterize the Hg²⁺-induced unregulated MPTP and to determine whether the reduced swelling seen in sucrose was due to a pore-size-dependent limit for permeation of the sugar, the size-exclusion limit of the unregulated MPTP was estimated with PEGs of different molecular sizes in KCl-based buffer (Fig. 7). The apparent size-exclusion limit was estimated to be 540 Da. This value is substantially lower than the size exclusion typically reported for the regulated mammalian pore (~1,500 Da). Furthermore, if KCl was replaced by sucrose at a constant overall osmolarity of 350 mosM, swelling was inhibited as the concentration of sucrose was increased (Fig. 7, inset). At 100 mM sucrose, the reduction in Hg²⁺-induced swelling was 51.7 ± 1.53% (SD) (n = 3). At the same sucrose concentration with rat mitochondria, the inhibition of swelling was significantly lower [25.0 ± 2.2% (SD); n = 3, P < 0.05], which is consistent with the larger pore size. For *A. franciscana* mitochondria, the larger effect of sucrose (molecular weight = 342) on swelling, compared with PEG of similar molecular weight (molecular weight = 400, Fig. 7), likely was due to the different shapes of the molecules (see DISCUSSION; Evidence for a nonregulated MPTP and its properties).

Finally, as predicted from the above results, when sucrose under isosmotic conditions (as in Fig. 5C; change in absorbance in response to mercury = 0.07) was replaced isosmotically with potassium chloride (400 mM KCl and 20 mM K-HEPES, pH 7.5, 880 mosM), a much greater swelling occurred in response to mercury [change in absorbance = 0.48 ± 0.03 (SD), n = 3]. Thus inhibition of swelling in sucrose-based medium clearly can be attributed to a pore-size-dependent exclusion of the sugar.

**Influence of high calcium and mercury on cytochrome c release.** The incubation of energized mitochondria from *A. franciscana* (0.5 mg protein/ml, in 500 mM sucrose, 150 mM KCl, 1 mM KH₂PO₄, 5 μM rotenone, 25 μM EGTA, and 20 mM K⁺-HEPES) with 1 mM calcium for 30 min did not induce release of cytochrome c [Fig. 8; iso Hg²⁺]. Also, no release could be detected from energized mitochondria after addition of 20 μM HgCl₂ (Fig. 8, iso Hg²⁺), although a slight swelling was previously noted with this treatment (Fig. 6C). This small increase in volume was apparently insufficient to cause mitochondrial outer membrane permeabilization. In contrast, when mitochondria were incubated in sucrose-free, hyposmotic solution (350 mosM containing 150 mM KCl and 20 mM K⁺-HEPES, pH 7.5) and 20 μM HgCl₂ was added, a major release of cytochrome c was observed (Fig. 8, hypo Hg²⁺), fully consistent with the large swelling induced by this treatment (Fig. 6E). Because of the reduced osmolarity of this medium, a small amount of cytochrome c release was also seen in controls (Fig. 8, hypo).

**DISCUSSION**

We have shown in this study that mitochondria from *A. franciscana* exhibit a remarkable calcium storage capability. A mercury-induced, nonregulated mitochondrial permeability transition was found in *A. franciscana*, but a regulated calcium

Fig. 5. Effect of 20 μM HgCl₂ on mitochondria isolated from 24-h nauplius larvae of *A. franciscana*. Control incubations of energized (trace A) and deenergized (trace B) mitochondria (0.5 mg protein/ml) in 500 mM sucrose, 150 mM KCl, 1 mM KH₂PO₄, 5 μM rotenone, 25 μM EGTA, and 20 mM K⁺-HEPES, pH 7.5, 920 mosM, result in stable volumes during the time monitored. Additions of 20 μM HgCl₂ to deenergized (trace C) or energized (trace D) mitochondria in the above medium cause slow decreases in absorbance (i.e., slight swelling).

Fig. 6. Effect of nonphysiological MPTP inducers on mitochondria isolated from 24-h nauplius larvae of *A. franciscana*. Deenergized mitochondria were incubated in sucrose-free, hyposmotic buffer (150 mM KCl and 20 mM K⁺-HEPES, pH 7.5, 350 mosM) to enhance swelling responses (see text). Changes in mitochondrial volume were monitored after addition of 50 μM atracyloside, 5 mM phosphate, and 0.1 mM CaCl₂ (trace A); 1 mM mstopharan and 5 mM phosphate (trace B); no additions (trace C); 100 μM phenylarsine oxide, 5 mM phosphate, and 0.1 mM CaCl₂ (trace D); 20 μM HgCl₂ (trace E); and 20 μM HgCl₂ plus 1 μM cyclosporin A (trace F).
As a result of calcium overload in rat mitochondria, MPTP opening and release of previously accumulated calcium from the matrix occur after addition of 0.1 mM CaCl$_2$ (Figs. 2B and 3C), a phenomenon that has been described as one of the prerequisites for programmed cell death (45, 46). In contrast, mitochondria from *A. franciscana* are able to actively load calcium even when challenged with 1 mM extramitochondrial calcium (Fig. 2A). This remarkable capacity of calcium uptake and storage was measured in the absence of Mg$^{2+}$ and exogenous nucleotides, conditions that favor mitochondrial permeability transition in mammalian systems. No release of previously accumulated calcium from the matrix occurs in *A. franciscana* (Fig. 2A). Furthermore, after addition of 1 mM calcium to energized mitochondria from *A. franciscana*, an apparent decrease in mitochondrial volume (increase in absorbance) can be observed (Fig. 3A). The apparent shrinkage of mitochondria from *A. franciscana* was a specific response to calcium; none was found in response to 1 mM MgCl$_2$, and the calcium effect was abolished in phosphate-free medium (data not shown). Such an apparent shrinkage of mammalian mitochondria, measured by absorbance during matrix calcium loading, is believed to be caused by the formation of calcium-phosphate complexes in the matrix. These complexes cause an increase in the refractive index of the matrix, and the observed change in absorbance is not thought to represent a decrease in mitochondrial volume (2, 13, 58).

**Evidence for a nonregulated MPTP and its properties.** Changes in mitochondrial volume were not found after the addition of 50 μM atracyloside, 5 mM phosphate, and 0.1 mM CaCl$_2$; 1 mM mastoparan and 5 mM phosphate; or 100 μM phenylarsine oxide, 5 mM phosphate, and 0.1 mM CaCl$_2$ (Fig. 6A, B, D) to energized mitochondria from *A. franciscana*. The presence of a regulated MPTP can therefore be excluded. However, a high-amplitude swelling was found after the addition of 20 μM HgCl$_2$ in sucrose-free hypotonic medium. This effect was cyclosporin A insensitive (Fig. 6E and F) and the size-exclusion limit was 540 Da, well below the value of 1,500 Da found for the mammalian pore (74). The addition of 100 mosM sucrose (molecular mass = 342 Da) led to a larger reduction in swelling than the addition of PEG-400 (Fig. 7). This greater than expected reduction with sucrose is likely due to a bigger radius of the heterodimeric sugar compared with the linear geometry of the PEGs.

![Diagram](http://ajpregu.physiology.org/)

**Fig. 7.** Solute size-exclusion properties of the mercury-induced permeability transition in mitochondria isolated from 24-h larvae of *A. franciscana*. Deenergized mitochondria were incubated at 0.5 mg/ml and 25°C in medium (pH 7.4) containing 150 mM KCl and prescribed amounts of polyethylene glycol (PEG); the total osmotic pressure was ~400 mosM, and 25% of this value was derived from PEG. PEGs ranging from 0.2 to 1.0 kDa were employed. Swelling was initiated by addition of 20 μM HgCl$_2$ and allowed to proceed until absorbance of the suspension was constant. These final values, expressed as a percentage obtained in the absence of PEG, are plotted as a function of the PEG molecular weight ($M_w$; as a percentage obtained in the absence of PEG, are plotted as a function of the $M_w$; sucrose [increasing the sugar concentrations at constant overall osmolarity), a progressive decrease in mercury-induced swelling is observed. Each value in inset is mean ± SD of $n=3$ experiments.

**Calcium uptake and the absence of a regulated MPTP.** In mammalian mitochondria, calcium distribution represents a steady state where electrophoretic calcium uptake via the calcium uniporter and/or rapid-uptake mode is matched by calcium efflux on two separate pathways: the sodium-dependent pathway and sodium-independent calcium efflux pathway (29). Because the maximal rate of calcium efflux via these pathways is much lower than that of calcium uptake, mitochondria exposed to high calcium are susceptible to the hazard of calcium overload (8). Mammalian mitochondria show a rapid swelling after matrix overload with calcium in the presence of P$_i$, which occurs with higher probability if the mitochondria are deenergized (35). As with rat mitochondria, mitochondria from *A. franciscana* exhibit an uptake of calcium as measured with the membrane-impermeable calcium indicator fluo-5N (Fig. 2).

As a result of calcium overload in rat mitochondria, MPTP opening and release of previously accumulated calcium from the matrix occur after addition of 0.1 mM CaCl$_2$ (Figs. 2B and 3C), a phenomenon that has been described as one of the prerequisites for programmed cell death (45, 46). In contrast, mitochondria from *A. franciscana* are able to actively load calcium even when challenged with 1 mM extramitochondrial calcium (Fig. 2A). This remarkable capacity of calcium uptake and storage was measured in the absence of Mg$^{2+}$ and exogenous nucleotides, conditions that favor mitochondrial permeability transition in mammalian systems. No release of previously accumulated calcium from the matrix occurs in *A. franciscana* (Fig. 2A). Furthermore, after addition of 1 mM calcium to energized mitochondria from *A. franciscana*, an apparent decrease in mitochondrial volume (increase in absorbance) can be observed (Fig. 3A). The apparent shrinkage of mitochondria from *A. franciscana* was a specific response to calcium; none was found in response to 1 mM MgCl$_2$, and the calcium effect was abolished in phosphate-free medium (data not shown). Such an apparent shrinkage of mammalian mitochondria, measured by absorbance during matrix calcium loading, is believed to be caused by the formation of calcium-phosphate complexes in the matrix. These complexes cause an increase in the refractive index of the matrix, and the observed change in absorbance is not thought to represent a decrease in mitochondrial volume (2, 13, 58).

**Evidence for a nonregulated MPTP and its properties.** Changes in mitochondrial volume were not found after the addition of 50 μM atracyloside, 5 mM phosphate, and 0.1 mM CaCl$_2$; 1 mM mastoparan and 5 mM phosphate; or 100 μM phenylarsine oxide, 5 mM phosphate, and 0.1 mM CaCl$_2$ (Fig. 6A, B, D) to energized mitochondria from *A. franciscana*. The presence of a regulated MPTP can therefore be excluded. However, a high-amplitude swelling was found after the addition of 20 μM HgCl$_2$ in sucrose-free hypotonic medium. This effect was cyclosporin A insensitive (Fig. 6E and F) and the size-exclusion limit was 540 Da, well below the value of 1,500 Da found for the mammalian pore (74). The addition of 100 mosM sucrose (molecular mass = 342 Da) led to a larger reduction in swelling than the addition of PEG-400 (Fig. 7). This greater than expected reduction with sucrose is likely due to a bigger radius of the heterodimeric sugar compared with the linear geometry of the PEGs.

![Diagram](http://ajpregu.physiology.org/)

**Fig. 8.** Western blot analysis of proteins released from mitochondria isolated from 24-h nauplius larvae of *A. franciscana*. Positive controls were cyt-c (purified horse heart cytochrome c) and mitochondria (supernatant from homogenized mitochondria). For experimental treatments, mitochondria were incubated as described below and centrifuged, and supernatants were analyzed. Energized mitochondria (incubated for 30 min in 500 mM sucrose, 150 mM KCl, 1 mM KH$_2$PO$_4$, 5 μM rotenone, 5 mM succinate, 25 μM EGTA, and 20 mM K-HEPES, pH 7.5) were without additions (iso (-)), plus 1 mM CaCl$_2$ (iso (+)), and plus 20 μM HgCl$_2$ (iso Hg$^{2+}$); energized mitochondria (incubated for 15 min in 150 mM KCl, and 20 mM K-HEPES, pH 7.5) were without additions (hypo) and plus 20 μM HgCl$_2$ (hypo Hg$^{2+}$). Each lane was loaded with 0.015 mg of supernatant protein, and blots were probed with cytochrome c antibody.
More than 30 mitochondrial carriers have been described (59), and mercury is known to interact with several of these, such as the aspartate/glutamate carrier and ANTs, which can transform the carrier properties to be more channel-like and nonspecific (19, 20). He and Lemasters (40) proposed a two-step mode of mercury-induced mitochondrial permeabilization: activation of the regulated MPTP by low mercury and unspecific “damage” of membrane proteins at higher mercury, leading to an unregulated pore. No swelling could be detected if mitochondria from A. franciscana were incubated with a low concentration of mercury (5 μM) in the presence of 0.1 mM calcium (data not shown). Because the mercury-induced permeability transition in A. franciscana was 1) induced by a rather high concentration (20 μM), 2) strongly inhibited by succrose, and 3) cyclosporin A insensitive (Figs. 6 and 7), we suggest that the pore is comparable to the unregulated form in mammalian species.

Apoptotic signaling, anoxia tolerance, and the distribution of MPTPs. In mammals, the opening of the MPTP is associated with mitochondrial depolarization and the release of cytochrome c, which stimulates caspase-dependent apoptosis by binding to Apaf-1 (26, 27, 41, 55). The molecular mechanisms of programmed cell death are conserved during evolution, and recent evidence suggests the involvement of cytochrome c in apoptosis of some invertebrates. The Apaf-1 homolog CED-4 in C. elegans does not exhibit WD-40 repeats, which are necessary for the cytochrome c-mediated caspase activation (48); it is converted to an active form when the association between CED-4 and CED-9 is disrupted by EGL-1 (4, 46). In contrast, a cytochrome c-dependent apoptotic system that leads to caspase activation was found in Drosophila (46, 48), and cytochrome c-induced caspase-3-like activity was found in the oyster Crassostrea virginica (65). The presence of a cytochrome c-dependent apoptotic system in A. franciscana requires further investigation.

The high calcium tolerance of liver mitochondria from the great green goby (Zosterisessor ophiocephalus) was speculated to be associated with the greater hypoxia tolerance of this fish compared with mammalian species (67). In the hypoxia-tolerant oyster C. virginica, no cadmium-induced mitochondrial permeabilization could be found (65), but the effect of the physiological inducers calcium and Pi, was not investigated. We speculate that the inability to induce a regulated MPTP in A. franciscana might play a role in the ability of this animal to survive environmental insults and may hold true for higher invertebrates in general.

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