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Mitochondrial Ca^{2+} transport and permeability transition in zebrafish (*Danio rerio*)

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ABSTRACT

We have studied mitochondrial Ca^{2+} transport and the permeability transition (PT) in the teleost zebrafish (*Danio rerio*), a key model system for human diseases. Permeabilized zebrafish embryo cells displayed a mitochondrial energy-dependent Ca^{2+} uptake system that, like the Ca^{2+} uniporter of mammals, was inhibited by ruthenium red. Zebrafish mitochondria underwent a Ca^{2+}-dependent PT that displayed Pi-dependent desensitization by cyclosporin A, and responded appropriately to key modulators of the mammalian PT pore (voltage, pH, ubiquinone 0, dithiol oxidants and cross linkers, ligands of the adenine nucleotide translocator, arachidonic acid). Opening of the pore was documented in intact cells, where it led to death that could largely be prevented by cyclosporin A. Our results represent a necessary step toward the use of zebrafish for the screening and validation of PTP inhibitors of potential use in human diseases, as recently shown for collagen VI muscular dystrophy [Telfer et al., 2010].

1. Introduction

The Permeability Transition (PT) [1] is increasingly recognized as an effector mechanism of cell death [2,3]. The PT is an inner membrane permeability increase to solutes with molecular mass up to about 1500 Da, which leads to membrane depolarization, deregulation of ion homeostasis, depletion of pyridine nucleotides and ATP hydrolysis; PT-dependent cristae junction remodeling and matrix swelling favor cytochrome *c* release [4], which is maximal if outer membrane rupture takes place [5]. The PT is highly regulated, and it is presumed to occur through opening of a protein channel, the PT pore (PTP). In spite of major efforts the molecular nature of the PTP remains unknown [2,3]. The only protein involved in the PT identified so far is cyclophilin (CyP) D [6–8], the matrix receptor for cyclosporin (Cs) A [9–12]. We think that CyPD sensitizes the PTP to the inducing effects of Ca^{2+} and of other agonists by shielding an inhibitory site for Pi; genetic ablation of CyPD or its inhibition by CsA allows PTP desensitization by Pi, an effect that is lost when Pi is replaced by other anions like its analogs arsenate (Asi) and vanadate, or by bicarbonate (see ref. [13] and discussion therein). Mice with genetic inactivation of CyPD have been used to address the role of the PTP in disease, but conclusions based on this genotype must be taken with great caution because a PT still occurs in CyPD-null mitochondria, or in CyPD-competent mitochondria treated with CsA [14–17]. Indeed, and as we have noted before [15], CyPD-null animals can only address the role of CyPD, not of the PTP, in pathophysiology; and it is hard to make predictions on whether conditions allowing CyPD-dependent modulation of the PTP (specifically, an adequate concentration of Pi) will occur in specific disease paradigms.

The above considerations highlight the need for the development (i) of selective inhibitors of the PTP acting at sites different from CyPD and (ii) of model disease systems for the PTP that could be amenable to the rapid testing of drugs identified by high-throughput screening. We have provided the proof of principle that CyPD-independent sites exist with the discovery of the PTP inhibitory properties of quinones and of Ro 68-3400, which indeed displayed additive effects with those of CsA [18–22].

The vertebrate teleost zebrafish (*Danio rerio*) has recently become an attractive tool to uncover the complicated pathways regulating the biological processes that underlie several human diseases [23]. The importance of this model to PTP studies is dramatically highlighted by the recent findings that Ullrich Congenital Muscular Dystrophy (UCMD) can be modeled in zebrafish [24]; and that, like the mouse model [25–27], it can be cured with CsA through PTP inhibition [24]. Here, we have carried out a thorough characterization of mitochondrial Ca^{2+} transport and the PT in zebrafish. We show that zebrafish mitochondria readily undergo a Ca^{2+}-dependent PT that displays a Pi-dependent desensitization by CsA, and that responds appropriately to key modulators of the mammalian PTP.

Abbreviations: Asi, arsenate; CRC, calcium retention capacity; Cs, Cyclosporin; CyP, Cyclophilin; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; MOPS, 4-morpholinopropane sulfonic acid; PTP, permeability transition pore; TMRM, tetramethylrhodamine methyl ester; Ub0, ubiquinone 0

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2. Materials and methods

2.1. Animals

Adult zebrafish were maintained in the facility of University of Padova containing aerated, 28.5 °C-conditioned saline water according to standard protocols. Fish were kept under a 14-h light–10-h dark cycle. For mating, males and females were separated in the late afternoon and the next morning were freed to start courtship, which ended with eggs deposition and fecundation. Eggs were collected, washed with fish water (0.5 mM NaH_2PO_4, 0.5 mM $NaHPO_4$, 0.2 mg/l methylene blue, 3 mg/l instant ocean), and maintained at 28.5 °C in fish water supplemented with an antibiotic-antimycotic cocktail (50 μg/ml ampicillin, 100 units/ml penicillin and 0.1 mg/ml streptomycin, Biochrom, 3.3 μg/ml amphotericin B, Bristol-Myers-Squibb).

2.2. Isolation of cells from zebrafish embryos

Zebrafish cells were prepared from 6-h-old embryos (gastrula stage). To minimize bacterial contamination the antibiotic-antimycotic cocktail was included in all solutions used. Embryos were treated with 0.1% sodium hypochlorite in Holtfreter's solution (15 mM NaCl, 0.17 mM KCl, 0.23 mM $CaCl_2$, and 0.6 mM $NaHCO_3$, pH 7.6), abundantly washed with Holtfreter's solution, dechorionated and dissociated in 0.25% trypsin/EDTA by pipetting for 2 min. Chorions and undissociated embryos were separated from the cell suspension with a filter, while the embryonic cell suspension was diluted with serum-containing medium and centrifuged at 800*g* for 3 min. Cells were used immediately or plated in zebrafish growth medium (Leibovitz-15 medium L-15, Gibco), supplemented with 3% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 3.3 μg/ml amphotericin B on poly-D-lysine-coated supports (UV-sterilized 24-mm diameter glass coverslips, plastic flasks or wells).

2.3. Measurements on permeabilized cells

Freshly dissociated cells from embryos were washed in 130 mM KCl, 10 mM Tris-MOPS, 1 mM P_i-Tris, pH 7.4 and then incubated with 100 μM digitonin in the same buffer supplemented with 1 mM EGTA-Tris for 10 min. Digitonin was then washed away by spinning cells at 800*g* in 130 mM KCl, 10 mM Tris-MOPS. Measurements of membrane potential, extramitochondrial Ca^{2+}, and PN oxidation-reduction status were carried out fluorimetrically with Fluoroskan Ascent FL (Thermo Electron Corporation) on permeabilized cell suspension at 25 °C in the presence of 5 mM glutamate-Tris and 2.5 mM malate-Tris. Experiments were performed in 96-well black plates in a volume of 100 μl per well. Unless otherwise specified, the incubation buffer was 130 mM KCl, 10 mM Tris-MOPS, 1 mM P_i-Tris, 10 μM EGTA-Tris, pH 7.4. Membrane potential was followed based of the fluorescence changes of 0.5 μM rhodamine 123 (Molecular Probes) with the filter couple λ_{exc} 485 nm, λ_{em} 525 nm. In parallel, the PN oxidation-reduction status was evaluated based on endogenous NAD(P)H fluorescence (filter couple: 355–460 nm). Extramitochondrial Ca^{2+} concentration was measured in the presence of 0.5 μM Ca^{2+}-Green 5 N (Molecular Probes) with the filter couple λ_{exc} 485 nm, λ_{em} 525 nm.

2.4. Measurements of mitochondrial membrane potential in intact cells

Cellular fluorescence images were acquired at room temperature with an Olympus IX71/IX51 inverted microscope equipped with a digital camera. Adherent cells were first washed with phosphate-buffered saline and then incubated for 30 min at room temperature in serum-free L-15 medium supplemented with 4 μM CsH (which inhibits the plasma membrane multi-drug resistance P-glycoprotein, but not the PTP) and, when specified, 1.6 μM CsA or 0.6 μM FK506. Mitochondrial membrane potential was followed based on the accumulation of 10 nM tetramethylrhodamine methyl ester (TMRM, Molecular Probes) as previously described. For detection of TMRM fluorescence, 568 ± 25 nm bandpass excitation and 585 nm longpass emission filter settings were used. Sequential digital TMRM fluorescence images were collected every 3 min with exposure time of 100 ms (41.68% illumination intensity). Additions were performed between two acquisitions in the above medium and added volume never exceeded 1% of the incubation volume. At the end of each experiment, mitochondria were fully depolarized by the addition of the protonophore carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone (FCCP). Data were acquired and analyzed using the Cell R Software (Olympus). Clusters of several mitochondria were identified as regions of interest and fields not containing cells were used as background. Before each experiment, a light control was performed to ensure that light did not cause depolarization by itself.

2.5. Cell death assay

Occurrence of cell death was evaluated on the basis of accumulation of Sytox Orange (Molecular Probes). Cells were seeded on 24-well plates 2 days before the experiment. Adherent cells were washed with PBS and then incubated for 4 h at room temperature in serum-free L-15 medium with the compounds specified in the figure legends. When present, 0.6 μM FK506, 1.6 μM CsA or vehicle (DMSO) were pre-incubated for 30 min before further treatments. Cells were then incubated for 30 min with 0.2 μM Sytox Orange and total fluorescence intensities were acquired with the Fluoroskan Ascent FL apparatus. Fluorescence from wells containing medium plus Sytox Orange was considered as background. Digitonin was used as the fully permeabilizing agent, and Sytox fluorescence in digitonin-treated cells was taken as 100%.

2.6. Chemicals

All chemicals were of the highest purity commercially available, and unless stated otherwise they were from Sigma. Stock solutions were prepared according to the manufacturer's instructions.

3. Results

Preparation of mitochondria from zebrafish gave a low yield of organelles of poor quality. We therefore used digitonin-permeabilized cells from 6-h-old embryos (gastrula stage), an approach that allows measurements to be made with minimal disruption of mitochondrial integrity [28]. A 20 μM Ca^{2+} pulse was readily taken up by this preparation, but uptake was followed by spontaneous release of the accumulated cation (Fig. 1A, trace a). Treatment with CsA prevented Ca^{2+} release (Fig. 1A, trace b), indicating that it was due to PTP opening. Ca^{2+} uptake was prevented by pretreatment with ruthenium red, the classical inhibitor of the mitochondrial Ca^{2+} uniporter [29] (Fig. 1A, trace c). No further Ca^{2+} release could be seen upon inhibition of respiration with rotenone (Fig 1A). When Pi was replaced by Asi, onset of Ca^{2+} release occurred earlier, consistent with a slightly more pronounced PTP-inducing effect of Asi compared to Pi [13]. Note that the initial rate of Ca^{2+} uptake was the same in Pi- and Asi-containing media (compare traces a in panels A and B). This finding indicated that, like in liver mitochondria [13], maintenance and regeneration of the membrane potential was not affected by Asi. As expected [13], CsA was not able to prevent Ca^{2+} release in the Asi-containing medium while Ca^{2+} uptake was still inhibited by ruthenium red (Fig 1B).

Consistent with onset of a Ca^{2+}-dependent PT (i) addition of Ca^{2+} caused a profound mitochondrial depolarization (Fig. 2A, trace a) which was prevented by the addition of CsA, a condition under which only the expected, transient depolarization linked to Ca^{2+} uptake was observed (Fig. 2A, trace b); and (ii) the pyridine nucleotide pool underwent full