

Protocols

A fluorescence-based technique for screening compounds that protect against damage to brain mitochondria

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Abstract

Mitochondrial failure to generate ATP can be due to damage to their membranes, which leads to release of solutes, e.g., pyridine nucleotides, from the mitochondrial matrix. We developed a highly sensitive fluorescence assay for detecting a pathologic increase in mitochondrial membrane permeability. The assay is based on coupled enzymatic reactions that produce hydrogen peroxide in the presence of the reduced or oxidized form of nicotinamide adenine dinucleotide (NADH/NAD). The hydrogen peroxide is a substrate for horseradish peroxidase that converts Amplex Red into highly fluorescent Resorufin. The assay is able to detect nanomolar levels of pyridine nucleotides in the medium. Calcium additions to isolated rat brain or liver mitochondria incubated in a potassium-based medium with added enzymes caused osmotic swelling, as detected with light scattering, and production of Resorufin, due to release of NADH/NAD. These events were blocked by cyclosporin A (CsA) or Bongkrekic acid (BKA), inhibitors of the mitochondrial permeability transition (MPT). These results indicate that the NADH/NAD release assay is a simple, reliable, and sensitive method for detecting mitochondrial damage and for screening of compounds that protect mitochondria from injury.

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Theme: Disorders of the nervous system

Topic: Ischemia

Keywords: Mitochondria; Membrane damage; Permeability transition; Amplex Red; Pyridine nucleotides; Resorufin

1. Type of research

- Isolation of brain mitochondria
- calcium-induced mitochondrial permeability transition (MPT)
- screening of MPT inhibitors
- detection of damage to mitochondrial membranes

2. Time required

Time required for isolation of mitochondria is approximately 75 min. The assay can be completed within 15 min.

3. Materials

3.1. Animals

One male Wistar rat weighing 280–300 g was used for each mitochondrial preparation. Mitochondria from other strains or species can be employed.

3.2. Chemicals

Alcohol dehydrogenase (ADH), NADH oxidase (NADH Ox), horseradish peroxidase (HRP), cyclosporin A (CsA), nicotinamide adenine dinucleotide (NAD), calcium chloride, D-mannitol, sucrose, EGTA, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonate) (HEPES), Tris (hydroxymethyl)aminomethane, bovine serum albumin were purchased from Sigma (St Louis). Amplex Red was obtained from Molecular Probes (Eugene, OR). Supra-pure KCL was obtained from Merck (Darmstadt,

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Germany). Bongkreic acid was obtained from Calbiochem. Percoll was purchased from Amersham Biosciences (Piscataway). The reagents for Lowry protein determination assay were obtained from Bio-Rad (Hercules, CA).

3.3. Solutions

Mitochondrial isolation buffer (MIB): 225 mM sucrose, 75 mM mannitol, 1 mM EGTA, 5 mM HEPES, pH 7.4 at 4 °C; for isolation of liver mitochondria, 1 mg/ml of bovine serum albumin (BSA) was added to MIB.

Percoll: a 100% stock solution contained 225 mM sucrose, 75 mM mannitol, 1 mM EGTA, 5 mM HEPES, and pH 7.4 at 4 °C. This solution was diluted with MIB to obtain final Percoll concentrations of 15%, 24%, and 40% (v/v).

The medium used for the measurements of pyridine nucleotides and mitochondrial swelling contained 125 mM KCL, 20 mM HEPES–Tris, 10 μM EGTA, 0.096% ethanol, pH 7.5 at 37 °C. Experiments with liver mitochondria were carried out at 25 °C. The assay was carried out in 2 ml volume of medium supplemented with 1.5 U/ml of ADH, 50 mU/ml NADH oxidase, 5 U/ml of HRP, and 20 μM Amplex Red.

3.4. Equipment

For preparation of mitochondria from rat brain and rat liver, tissue was homogenized with a Potter-Elvehjem Teflon pestle, glass vessel homogenizer. Centrifugation was performed with a Beckman high-speed centrifuge and a JA-21 rotor.

The fluorescence of Resorufin was measured using a Hitachi 2500 (Tokyo, Japan) fluorescence spectrometer.

The protein assay was carried out with a Beckman spectrophotometer DU 7500.

4. Detailed procedure

4.1. Isolation of nonsynaptic brain mitochondria

Nonsynaptic brain mitochondria were isolated with slightly modified methods according to Ref. [17] and see also Ref. [10]. Following decapitation, the rat brain was quickly removed and placed in ice-cold MIB. All subsequent homogenization and centrifugation steps were carried out at 4 °C. The cerebellum and underlying structures were removed, and the remaining material was minced with scissors in 5 ml of MIB. Solution in excess of that which covered the settled brain tissue was decanted and replaced with 8 ml of fresh MIB. This suspension was homogenized by hand using 10 up and down strokes. The homogenate was centrifuged at $1330 \times g$ for 3 min. The supernatant was decanted, and the pellet was resuspended in half of the original volume and centrifuged as above. The pooled supernatant was centrifuged at $21,200 \times g$ for 10 min. The subsequent pellet was resuspended in 15% Percoll (3.5 ml) and layered into a centrifuge tube containing a preformed two-step discontinuous gradient consisting of 3.7 ml of 24% Percoll on top of 1.5 ml of 40% Percoll. The gradient was centrifuged at $30,700 \times g$ for 8 min. The mitochondrial fraction, located at the interface between the bottom layers, was removed and diluted 1:4 in isolation medium and centrifuged at $16,700 \times g$ for 10 min. The supernatant was decanted and the pellet resuspended in 0.5 ml BSA solution (10 mg/ml in H₂O) then diluted 1:10 with MIB. The suspension was then centrifuged at $6900 \times g$ for 10 min. The final mitochondrial pellet was resuspended in 100 μl of isolation medium lacking EGTA.

4.2. Isolation of rat liver mitochondria

Liver mitochondria were isolated according to Ref. [12]. After the rats were sacrificed by decapitation, all lobes of the liver were removed and placed in ice-cold MIB. The liver was

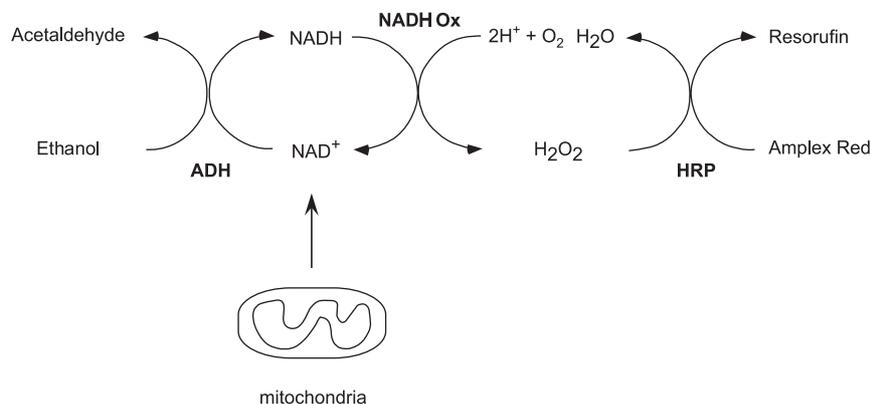


Fig. 1. Schematic diagram of enzymatic reactions used in the assay. When reduced or oxidized forms of nicotinamide adenine dinucleotides (NAD, NADH) are released from mitochondria due to damage to their membranes, alcohol dehydrogenase (ADH) in the presence of ethanol converts NAD to NADH, and ethanol is converted into acetaldehyde. NADH oxidase (NADH Ox) then oxidizes NADH to NAD and hydrogen peroxide is produced. In the final reaction, horseradish peroxidase (HRP) converts hydrogen peroxide to water coupled to the conversion of Amplex Red to the highly fluorescence compound Resorufin.

minced with scissors on ice and homogenized using four up and down strokes with a motor-driven pestle rotating at 550 rpm. The homogenate was centrifuged at $600 \times g$ for 12 min. The pellet was then discarded, and the supernatant was collected and centrifuged at $6800 \times g$ for 12 min. The supernatant was removed and the pellet resuspended in MIB and centrifuged again at $12,000 \times g$ for 12 min. The supernatant was again discarded and the pellet resuspended in MIB. After centrifugation at $12,000 \times g$ for 12 min, the final pellet as resuspended in a 1 ml of MIB without EGTA.

4.3. Protein assay

Mitochondrial protein was determined by the method of Lowry et al. [14] using Bio-Rad DC protein assay reagents.

The samples were measured in duplicates at 750 nm. Bovine serum albumin was used as standard.

4.4. Assay for pyridine nucleotides measurement

The assay for pyridine nucleotides measurement comprises several enzymatic reactions which use oxidized or reduced forms of nicotinamide adenine dinucleotides (NAD, NADH) as substrate, and the final product is the highly fluorescent compound Resorufin (see Fig. 1). The assay is performed in an isotonic, KCL-based medium maintained at 37° (see 3.3 Solutions). In the first step, alcohol dehydrogenase (ADH) catalyzes the oxidation of ethanol to form acetaldehyde, driving the reduction of NAD to NADH. The NADH formed is oxidized by

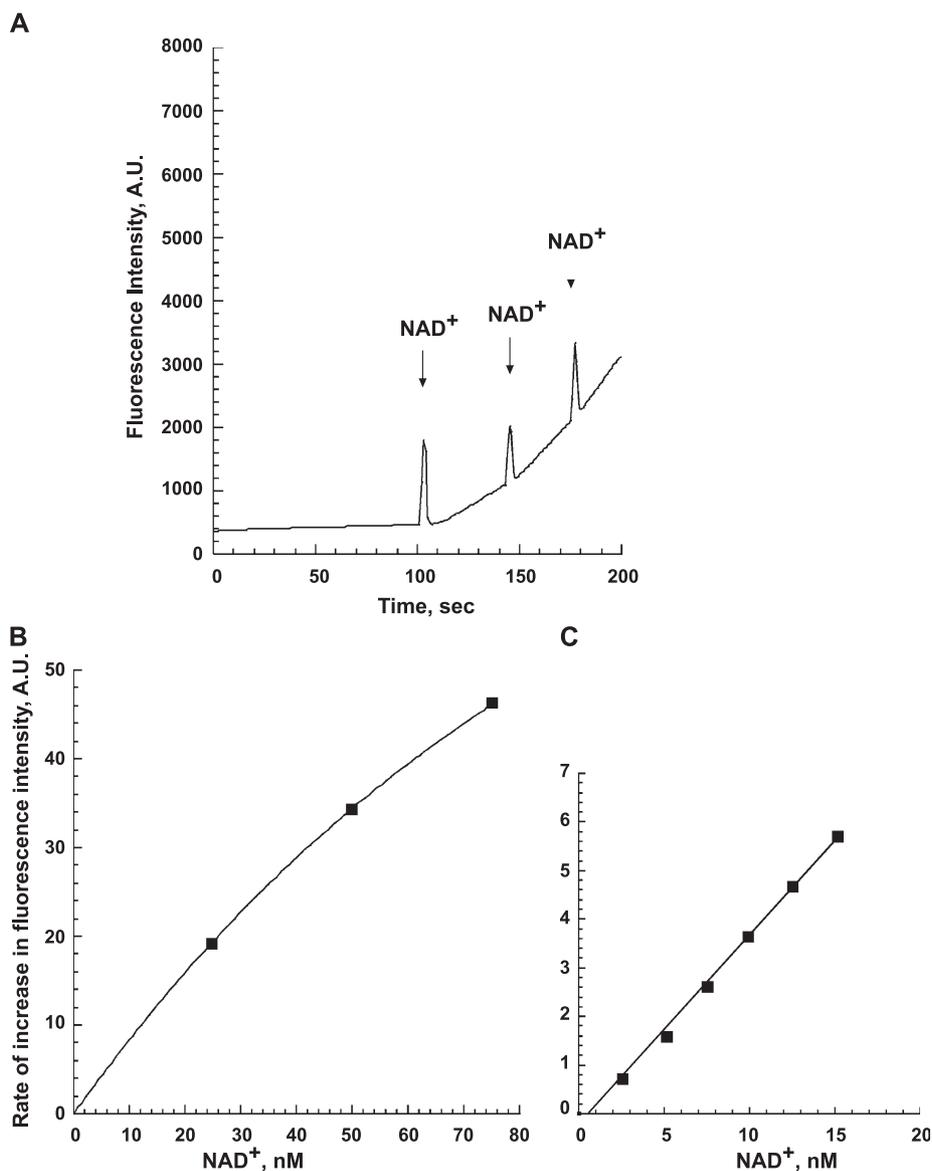


Fig. 2. Dose-response relationships between Resorufin fluorescence and NAD concentration using the enzyme-linked assay. (A) Sequential NAD additions (25 nmol each); (B) quantitative relationship between the increase in the rate of fluorescence and the concentration of added NAD; (C) experiments were carried out exactly as in (B), except the pulses of NAD were 2.5 nmol.

NADH oxidase, generating a stoichiometric amount of hydrogen peroxide (H_2O_2 ; [15]). Finally, in the presence of horseradish peroxidase (HRP), the H_2O_2 drives the oxidation of Amplex Red forming Resorufin, the fluorescent product (see Fig. 1). The Resorufin fluorescence is measured using an excitation wavelength of 550 nm and an emission wavelength of 585 nm. A stock solution of NAD of 50 μM was prepared in deionized water and used for calibration.

4.5. Measurement of mitochondrial swelling

Mitochondrial swelling was assessed from the changes in light scattering at 540 nm (for both excitation and emission wavelengths) measured in mitochondrial suspensions (0.125 mg protein/ml) using the same buffer as for the pyridine nucleotides assay. Each experiment was terminated by the

addition of alamethicin (40 $\mu\text{g}/\text{mg}$ protein), a nonselective pore-forming molecule, to induce maximal swelling of the whole mitochondrial population (see Refs. [10 11]). In the figures, light intensity after alamethicin treatment represents 0, and the light intensity before Ca^{2+} addition is marked as 100 on the ordinate.

5. Results and discussion

Repeated additions of NAD to the final assay mixture caused an incremental increase in the rate of Resorufin generation (Fig. 2A). Fig. 2B shows the relationship between NAD concentration in the medium and the rate of fluorescence increase. The background rate of fluorescence increase is very low, and the assay is highly sensitive, detecting nanomolar concentrations of pyridine nucleotides (Fig. 2C).

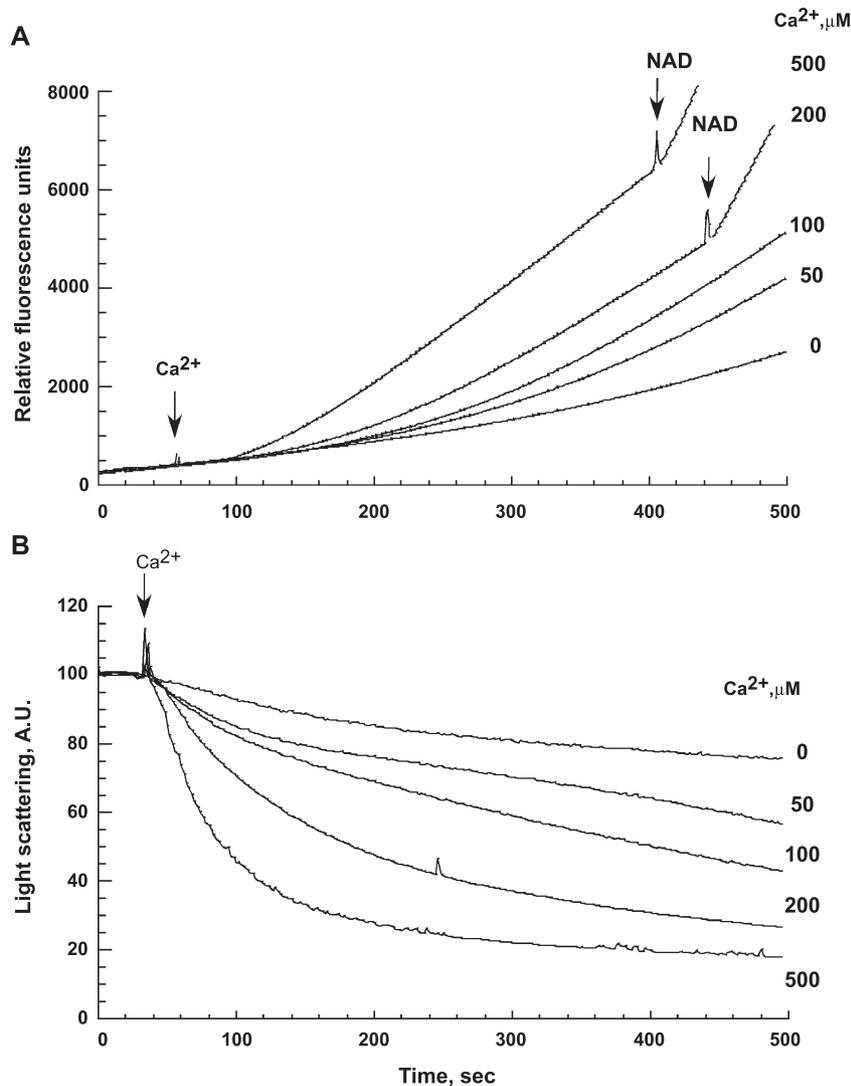


Fig. 3. Resorufin production (A) and changes in light scattering (B) induced by calcium additions to nonsynaptic rat brain mitochondria. Amount of added calcium is indicated at the individual traces (0, 50, 100, 200, and 500 μM Ca). In order to show that the fluorescence assay is not saturated when 200 or 500 μM Ca is used, 25 nmol of NAD was added at the end of the recording. A.U., arbitrary units.

Experiments performed with isolated brain mitochondria were conducted in the absence of respiratory substrates, as respiring mitochondria generate superoxide and subsequently H_2O_2 , which would interfere with the assay. As Figs. 3 and 4 demonstrate, in the absence of respiratory substrates, the background fluorescence is low, as has been previously demonstrated using Amplex Red detection of mitochondrial H_2O_2 production [5]. The fluorescence intensity did increase however, after the addition of calcium. This increase was not due to stimulation of H_2O_2 production by mitochondria, because calcium addition did not induce Resorufin generation when NADH oxidase was omitted from the medium (data not shown).

Addition of calcium induces opening of a membrane permeability transition (MPT) pore, a high-conductance, nonspecific channel in the mitochondrial inner membrane that is permeable to solutes up to 1.5 kDa in size (for review, see Refs. [8,1]). The opening of the MPT pore leads to osmotic swelling of mitochondria, which results in decreased light scattering. As Fig. 3 (lower panel) shows, calcium additions to brain mitochondria suspension induced a dose-dependent decrease in light scattering.

For screening purposes, it is often more convenient and efficient to use mitochondria isolated from liver, as the isolation procedure does not require gradient centrifugation, and the yield of mitochondrial protein from liver tissue is several-fold higher than from brain. Fig. 4 shows the changes in fluorescence intensity when different amounts

of calcium were added to liver mitochondria incubated in the presence of assay mixture. As with brain mitochondria, addition of calcium to liver mitochondria caused a progressive increase in Resorufin fluorescence intensity (see Figs. 4 and 5). This calcium-induced increase in fluorescence was inhibited by CsA (1 μM) and BKA (20 μM), examples of potent MPT pore inhibitors (Fig. 5, upper panel). CsA inhibits the MPT, most likely by binding to cyclophilin D, a matrix protein, which is the endogenous MPT modulator [4]. BKA inhibits the MPT by binding to the adenine nucleotide translocator [13], which modulates the MPT indirectly (for review, see Ref. [2]).

As Fig. 5 demonstrates, calcium addition induced an initial small drop in light scattering, which was followed by a progressive decrease starting at about 300 s following the calcium pulse. At the same time, there was a clear increase in Resorufin production (Fig. 5), indicating that the swelling was accompanied by release of pyridine nucleotides from mitochondria. These phenomena were inhibited by both CsA and BKA, indicating that the pathologic increase in mitochondrial membrane permeability was due to MPT pore opening. These results are in accordance with data reported by Di Lisa et al. [6], indicating that cardiac ischemia/reperfusion results in mitochondrial NAD depletion that can be blocked by treatment with CsA.

Another screening assay for detecting MPT was reported by Blattner et al. [3]. This approach requires loading of mitochondria with several fluorescent dyes in order to

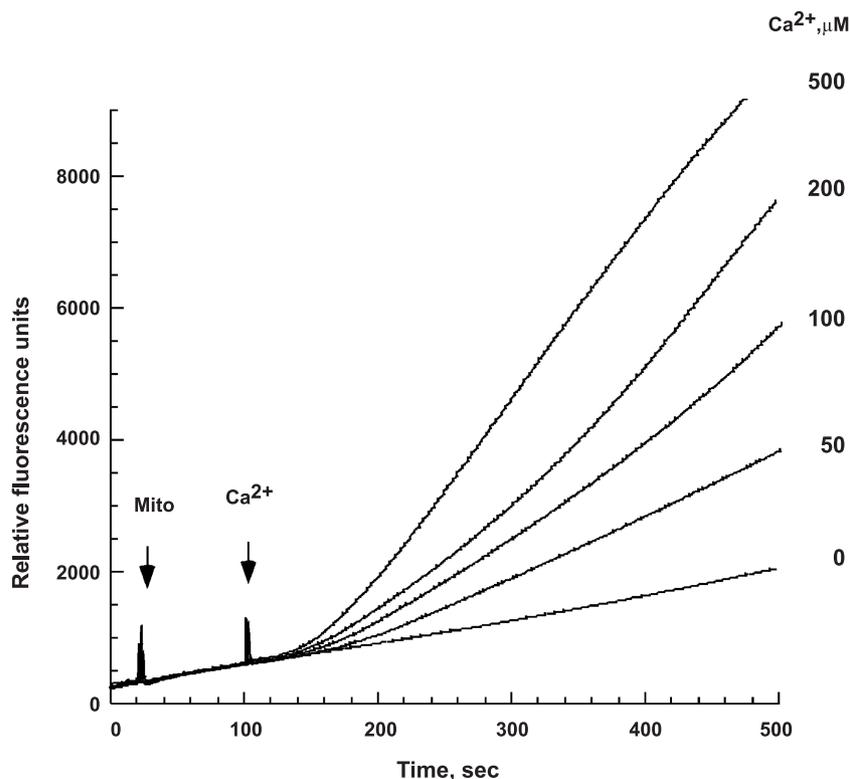


Fig. 4. Changes in fluorescence intensity due to calcium additions to liver mitochondria incubated with the assay mixture. Concentrations of calcium are indicated at the individual traces (0, 50, 100, 200, and 500 μM).

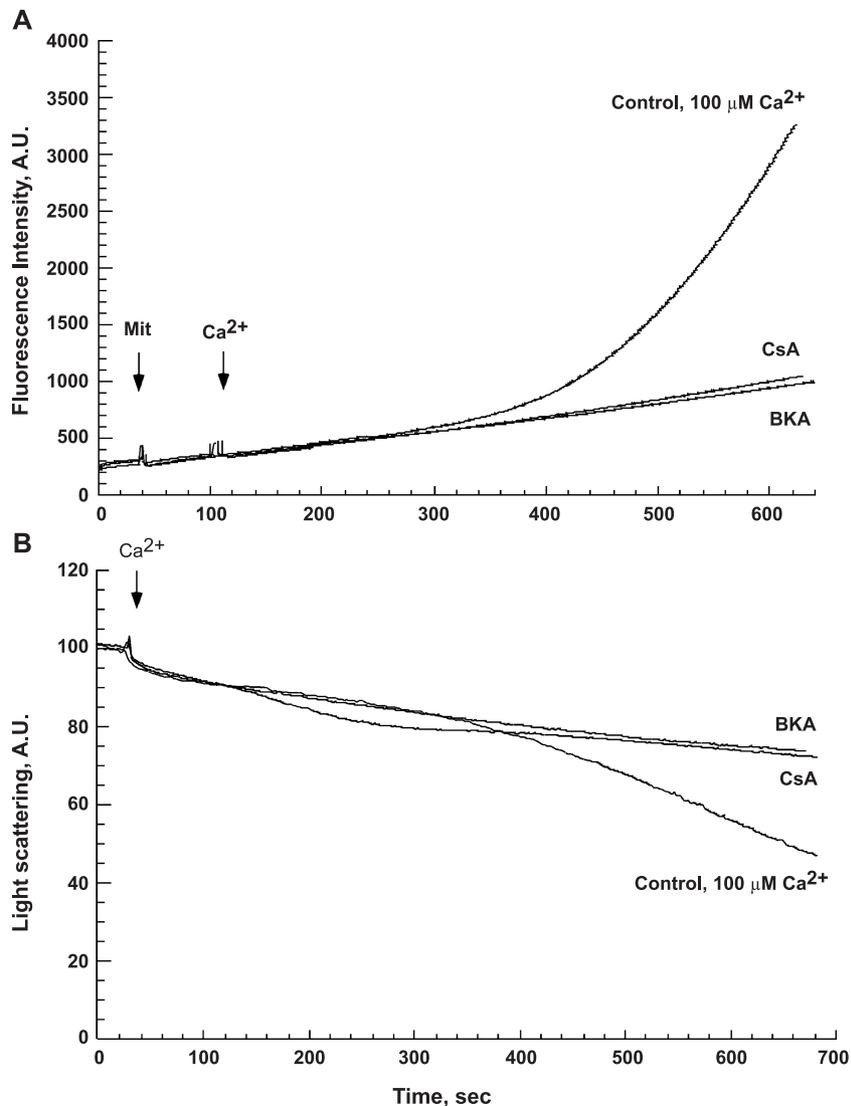


Fig. 5. Changes in Resorufin fluorescence intensity (A) and light scattering (B) following calcium additions to isolated liver mitochondria. Control: only calcium (100 μM) was added. BKA: the same amount of calcium was added to mitochondria incubated in the presence of 20 μM Bongkreikic acid. CsA: mitochondria were incubated in the presence of cyclosporin A (1 μM). The swelling is detected by recording the light scattering of the mitochondrial suspension at 540 nm. Decreased light scattering reflects swelling of mitochondria. A.U., arbitrary units.

distinguish MPT from mitochondrial respiratory uncoupling or inhibition and associated inhibition of calcium uptake. In their assay, isolated liver mitochondria are loaded with tetramethylrhodamine methyl ester (TMRM), a fluorophore that responds to mitochondrial membrane potential, and incubated in the presence of Fluo-5N, a low-affinity calcium indicator. Changes in TMRM and Fluo-5N are recorded simultaneously with swelling-induced light-scattering measurements following calcium additions. Another approach involves loading mitochondria with carboxydichlorofluorescein (DCF). The release of DCF from mitochondria due to calcium addition is correlated with swelling measurements. These assays therefore require several steps and the inclusion of chemicals in addition to calcium that can potentially affect the MPT. In contrast, our assay relies on the release of endogenous mitochondrial pyridine nucleotides triggered by

the sole addition of calcium in the absence of respiration that can complicate interpretation of results. However, in order to detect the effect of MPT inhibitors using the pyridine nucleotide release assay, a minimum incubation time of at least 5 min is required. For high-throughput screening, a well volume of approximately 0.2 ml could be used rather than the 2.0 ml volume used in the spectrofluorometer-based measurements. If the mitochondrial protein concentration is kept constant, then a 30 mg yield from one rat liver mitochondrial preparation would be sufficient for approximately 1000 assays. At 10–20 min per 96-well plate, these assays could be completed within 2 to 4 h.

In summary, drug development and discovery requires simple, reliable screening assays to identify agents that have specific effects. For example, CsA that inhibits the MPT can dramatically ameliorate brain damage due to

ischemic insult [19,20], furthermore this compound also shows a protective effect in brain damage induced by hypoglycemic coma [7], focal ischemia [21], and trauma ([18], for review, see Refs. [9,16]).

We have developed a fluorescence assay, which allows without any manipulation of isolated mitochondria, detection of damage to their membranes. For this reason, it is useful in discovering drugs that can protect mitochondria against calcium-related damage.

Acknowledgements

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