

# Mitochondrial oxidative stress induced by $\text{Ca}^{2+}$ and monoamines: different behaviour of liver and brain mitochondria in undergoing permeability transition

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**Abstract** Mitochondrial permeability transition (MPT) is correlated with the opening of a nonspecific pore, the so-called transition pore, that triggers bidirectional traffic of inorganic solutes and metabolites across the mitochondrial membrane. This phenomenon is caused by supraphysiological  $\text{Ca}^{2+}$  concentrations and by other compounds leading to oxidative stress, while cyclosporin A, ADP, bongkreic acid, antioxidant agents and naturally occurring polyamines strongly inhibit it. The effects of polyamines, including the diamine agmatine, have been widely studied in several types of mitochondria. The effects of monoamines on MPT have to date, been less well-studied, even if they are involved in a variety of neurological and neuroendocrine processes. This study shows that in rat liver mitochondria (RLM), monoamines such as tyramine, serotonin and dopamine amplify the swelling induced by calcium, and increase the oxidation of thiol groups and the production of hydrogen peroxide, effects that are counteracted by the above-mentioned inhibitors. In rat brain mitochondria (RBM), the monoamines do not amplify calcium-induced swelling, even if they demonstrate increases in the extent of oxidation of thiol groups and

hydrogen peroxide production. In these mitochondria, the antioxidants are not at all or scarcely effective in suppressing mitochondrial swelling. In conclusion, we hypothesize that different mechanisms induce the MPT in the two different types of mitochondria evaluated. Calcium and monoamines induce oxidative stress in RLM, which in turn appears to induce and amplify MPT. This process is not apparent in RBM, where MPT seems resistant to oxidative stress.

**Keywords** Mitochondria ·  $\text{Ca}^{2+}$  · Monoamines · Oxidative stress · Mitochondrial permeability transition pore

## Abbreviations

AdNT	Adenine nucleotide translocase
BHT	Butyl hydroxy toluene
BKA	Bongkreic acid
CsA	Cyclosporin A
CyP-D	Cyclophilin D
DA	Dopamine
DTE	Dithioerythritol
DTNB	5,5'-Dithio-bis (2-nitrobenzoic acid)
$\Delta\Psi$	Electrical transmembrane potential
MAO	Monoamine oxidase
MPT	Mitochondrial permeability transition
NEM	<i>N</i> -Ethylmaleimide
PTP	Permeability transition pore
RBM	Rat brain mitochondria
RLM	Rat liver mitochondria
ROS	Reactive oxygen species
SER	Serotonin
TPP <sup>+</sup>	Tetraphenylphosphonium
TXT	Toloxatone
TYR	Tyramine

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## Introduction

Intrinsic apoptosis is a mitochondrial mediated form of cellular death that can be induced in all mammalian cells. It is triggered by the occurrence of the mitochondrial permeability transition (MPT), an event correlated with the assembly of particular membrane proteins, leading to the opening of the so-called permeability transition pore (PTP). The subsequent release of specific mitochondrial factors that activate the caspase cascade and endonucleases result in DNA damage and apoptotic cell death (Orrenius et al. 2003).

These pro-apoptotic factors, released from isolated mitochondria, follow the rupture of the outer membrane as a result of a large colloid-osmotic swelling of the organelles. This event is induced by the opening of PTP, generally caused by supraphysiological  $\text{Ca}^{2+}$  levels and by additional factors leading to oxidative stress. The opening of PTP triggers bidirectional traffic of solutes and metabolites across the mitochondrial membrane and in particular, the release of cations, adenine and oxidized pyridine nucleotides and glutathione. Several proteins have been proposed to be either structural or regulatory components of the PTP, including the ATP/ADP translocator, the phosphate carrier, and cyclophilin-D (CyP-D) (Halestrap 2009). A widely held model places the adenine nucleotide translocase (AdNT) as the pore-forming component in the inner mitochondrial membrane, with peptidyl-prolyl *cis-trans* isomerase activity of CyP-D, facilitating a calcium-induced conformational change. The role of AdNT in PTP formation was confirmed by the activatory effects displayed by agents such as carboxyatractyloside that induce the “c” conformation (open pore) of the AdNT and the inhibitory effects by agents such as bongkreikic acid (BKA) that enhance the “m” conformation (closed pore). This model was initially proposed by Halestrap and Davidson (1990) but recently the same group suggested that AdNT is not the main pore-forming component, but rather a regulator component of PTP (Leung et al. 2008).

The established protective agents against MPT include the immunosuppressant cyclosporin A (CsA), ADP, the ATP/ADP translocator inhibitor bongkreikic acid (BKA), and antioxidant agents. Naturally occurring polyamines such as spermine, spermidine, putrescine, are also potent inhibitors of PTP opening as they are able to scavenge the highly toxic hydroxyl radical, responsible for the oxidative stress induction (Agostinelli et al. 2010). However, due to their polycationic nature, polyamines may exhibit their protection also by interacting with anionic charges located on the pore-forming structures (Tassani et al. 1995, Dalla Via et al. 2004).

The prevention on MPT induction by polyamines has been studied in isolated liver, heart, brain and kidney mitochondria (Lapidus et al. 1994). Other molecules

having amino groups also display suppression of MPT, one example being the propargylamines (Marcocci et al. 2002). Differences can occur depending on the organ of origin. For example, the biogenic amine agmatine is able to protect brain and kidney MPT (Arndt et al. 2009; Battaglia et al. 2010), but exhibits a concentration-dependent biphasic effect in rat liver mitochondria (RLM). At low concentrations (10–100  $\mu\text{M}$ ), in fact, it induces the MPT by means of increased oxidative stress caused by reactive oxygen species (ROS) produced as a by-product of agmatine catabolism (Battaglia et al. 2007). This fact is likely explained by the presence in RLM of a diamine oxidase able to oxidize agmatine, with the generation of  $\text{H}_2\text{O}_2$  and, most likely,  $\text{OH}^\cdot$  (Stevanato et al. 2011). At high concentrations ( $\sim 0.5$ –1 mM range) unreacted agmatine scavenges the ROS formed by itself and prevents MPT induction, thus exhibiting self protection.

Unlike the polyamines, the effects of monoamines on MPT induction or prevention in mitochondria of divergent organs have not been well investigated. It has been reported that tyramine and benzylamine are able to amplify the PTP opening induced by  $\text{Ca}^{2+}$  and phosphate in RLM (Marcocci et al. 2002). The dopamine reaction product, dopaquinone, showed similar effects (Bisaglia et al. 2010), while in other studies, dopamine appears not amplifying the MPT (Brenner-Lavie et al. 2008). These studies demonstrate the lack of a clear and common mechanism in response to monoamines in the different types of mitochondria. The aim of the present study was to evaluate the effect of monoamines such as dopamine and tyramine on MPT induction, and compare the outcomes in isolated RLM and rat brain mitochondria (RBM). We also evaluated the action of the monoamine serotonin, as little has been done in this model with this important neurotransmitter molecule. It might be worth mentioning that monoamines play an important role in various neurological and neuroendocrine processes and that neurons are monoamine producing cells, while mature hepatocytes do not normally produce this type of amino-containing compounds. A particular objective of this study was to evidence the different answer of RLM and RBM to the oxidative stress induced by monoamine oxidation; and it was found that the oxidative stress leads to MPT occurrence in RLM but not in RBM, thus demonstrating that different mechanisms control this phenomenon in these types of mitochondria.

## Materials and methods

### Isolation of RLM and RBM

Rat liver was homogenized in isolation medium (250 mM sucrose, 5 mM HEPES, 0.5 mM EGTA, pH 7.4) and

subjected to centrifugation ( $900 \times g$ ) for 5 min. The supernatant was centrifuged at  $12,000 \times g$  for 10 min to separate the crude mitochondrial pellets. The resulting pellets were suspended in isolation medium lacking EGTA (Schneider and Hogeboom 1953).

RBM were isolated by conventional differential centrifugation method and purified by the Ficoll gradient method, essentially according to Nicholls (1978), but with some modifications. Briefly: rat brain (cerebral cortex) was homogenized in isolation medium (320 mM sucrose, 5 mM HEPES, 0.5 mM EDTA, pH 7.4) supplemented with 0.3% BSA during homogenization and subjected to centrifugation ( $900 \times g$ ) for 5 min. The supernatant was then centrifuged at  $17,000 \times g$  for 10 min, to precipitate crude mitochondrial pellets. These were resuspended in isolation medium supplemented with 1 mM ATP and layered on top of the discontinuous gradient, composed of 2 ml of isolation medium containing 12% (w/v) Ficoll, 3 ml each of isolation medium containing 9 and 6% (w/v) Ficoll, respectively. The mitochondrial suspension and gradient were centrifuged for 30 min at  $75,000 \times g$ . Mitochondrial pellets were suspended in isolation medium and centrifuged for 10 min at  $17,000 \times g$ . The pellets were then suspended again in isolation medium without EDTA. Protein content was measured by the biuret method with BSA as standard (Gornall et al. 1949). These studies were performed in accordance with the guiding principles in the care and use of animals, and approved by the Italian Ministry of Health.

#### Standard incubation conditions for RLM and RBM

RBM and RLM (1 mg protein/ml) were incubated in a water-jacketed cell at  $20^\circ\text{C}$ . The standard medium contained 200 mM sucrose, 10 mM HEPES (pH 7.4), 5 mM sodium succinate, 1 mM sodium phosphate, and  $1.25 \mu\text{M}$  rotenone. 50 and  $100 \mu\text{M}$   $\text{Ca}^{2+}$  was added for the assays in RLM and RBM, respectively. Variations and/or other additions are described in the specific experiments presented.

#### Determination of mitochondrial swelling

Mitochondrial swelling was determined by measuring the apparent absorbance change of mitochondrial suspensions at a wavelength of 540 nm in a Kontron Uvikon model 922 spectrophotometer equipped with a thermostatic control.

#### Determination of mitochondrial functions

Electrical transmembrane potential ( $\Delta\Psi$ ) was calculated by determining the distribution of the lipid soluble cation tetraphenylphosphonium ( $\text{TPP}^+$ ) through the inner membrane,

measured by a  $\text{TPP}^+$  selective electrode prepared in our laboratory, according to previously published procedures (Kamo et al. 1979).

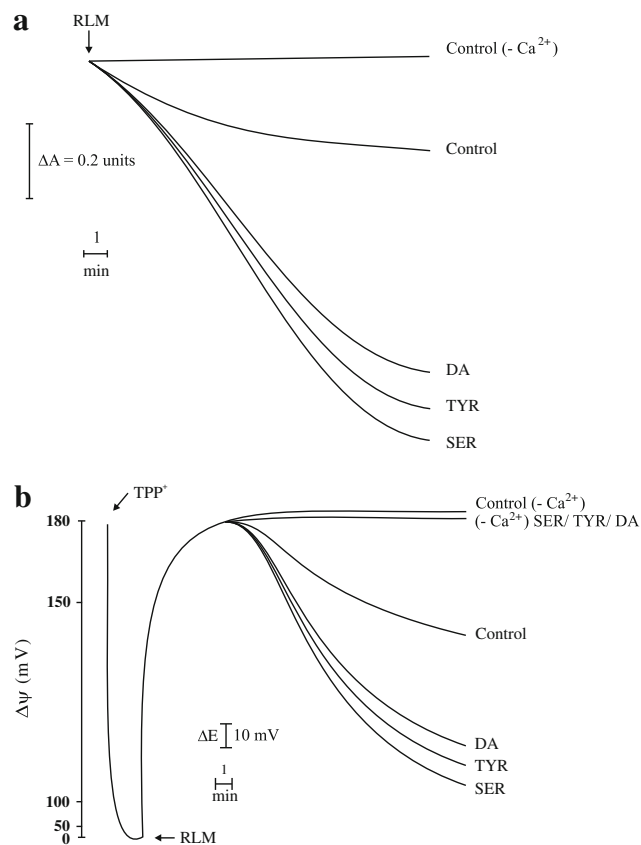
Protein sulfhydryl groups were determined with aliquots of mitochondrial suspensions taken from the incubated samples to evaluate mitochondrial swelling. Briefly, at the end of incubation (15 min), the total suspension (1 mg/ml) was placed in Eppendorf 4515c tubes and centrifuged for 1 min at  $12,000 \times g$ ; then the supernatant was discarded and the pellet used for both measurements. Sulfhydryl group oxidation assay was performed after solubilization of the pellet with 1 mg of solubilization medium (10 mM EDTA, 0.2 M Tris-HCl, 1% SDS, pH 8.3), with 5,5'-dithio-bis-(2-nitrobenzoic acid) at 412 nm in a Kontron Uvikon Model 922 spectrophotometer, according to Santos et al. (1998). Variations between samples were analyzed by ANOVA, with significance determined by Student's *t* test.

The redox state of endogenous pyridine nucleotides was directly followed fluorometrically in a Shimadzu spectrofluorophotometer RF-5,000, with excitation and emission wavelengths of 354 and 462 nm, respectively.

Mitochondrial  $\text{H}_2\text{O}_2$  release was assessed by the oxidation of Amplex Red ( $40 \mu\text{M}$ ) by horseradish peroxidase ( $15 \mu\text{g/ml}$ ) induced by  $\text{H}_2\text{O}_2$  (Boveris et al. 1977, Loschen et al. 1971). Amplex Red fluorescence was monitored at excitation and emission wavelengths of 544 and 590 nm, respectively, on a Fluoroskan Ascent FL plate reader (Mohanty et al. 1997) in 24-well plates maintained at  $37^\circ\text{C}$  with the indicated concentration of substrates. The reaction was started with the addition of the Amplex Red/HRP mixture, and the formation of the fluorescent product resorufin was detected. Values are reported as arbitrary units of fluorescence.

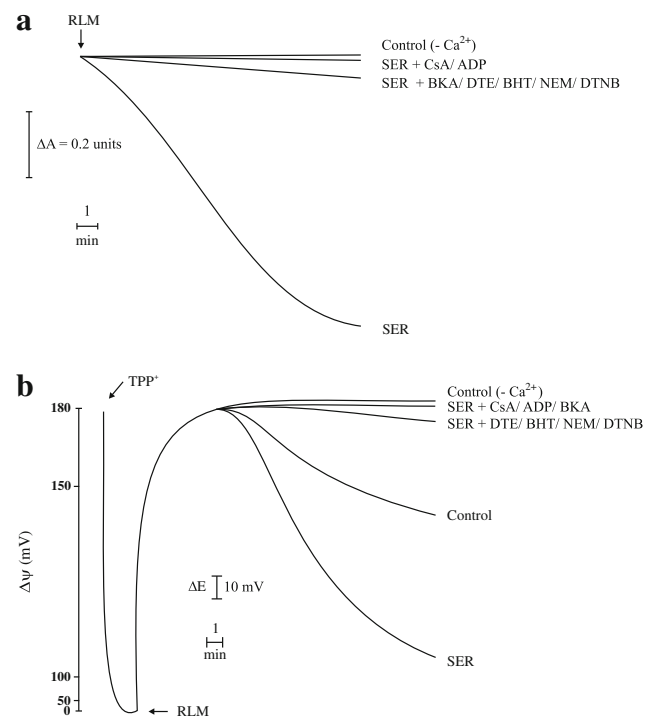
## Results

The results reported in Fig. 1a show that the suspensions of RLM, energized by the oxidation of succinate in the presence of rotenone, and incubated under the adopted experimental conditions with supraphysiological  $\text{Ca}^{2+}$  concentration ( $50 \mu\text{M}$ ) and phosphate, exhibit an apparent decrease in absorbance of about 0.200 AU/10 min. This absorbance decrease is indicative of mitochondrial colloid-osmotic swelling. The presence of the monoamines, such as, serotonin, tyramine, and dopamine causes a further marked absorbance decrease suggesting an increment of mitochondrial swelling. The above results are paralleled by a consistent  $\text{Ca}^{2+}$ -induced decrease in  $\Delta\Psi$ , from 180 mV (control value) to about 140 mV. In the presence of the monoamines the  $\Delta\Psi$  almost completely collapses (Fig. 1b). The large amplification of mitochondrial swelling and  $\Delta\Psi$  collapse induced by serotonin are significantly

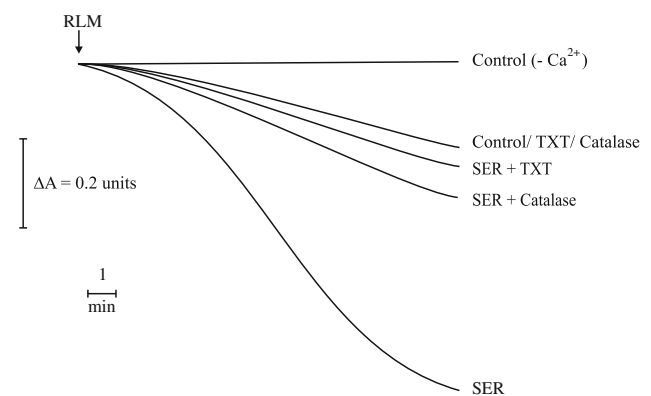


**Fig. 1** Effect of monoamines on mitochondrial swelling (a) and  $\Delta\Psi$  drop (b) induced by  $\text{Ca}^{2+}$  in RLM. RLM were incubated in standard medium as described in “Materials and methods”.  $50\ \mu\text{M}$   $\text{Ca}^{2+}$  was always present except when otherwise indicated; the concentration of added monoamines was  $100\ \mu\text{M}$ . Downward deflections indicate mitochondrial swelling (a).  $2\ \mu\text{M}$   $\text{TPP}^+$  was used for  $\Delta\Psi$  measurements.  $\Delta E$  is the electrode potential (b). Traces are representative of six separate experiments

inhibited by the typical MPT inhibitors, CsA, ADP, BKA, the antioxidant agents dithioerythritol (DTE) and butyl hydroxy toluene (BHT), by the SH-reagent 5,5'-Dithio-bis (2-nitrobenzoic acid) (DTNB) and by the alkylating agent *N*-ethylmaleimide (NEM) (Fig. 2a, b). Similar results have been obtained also with tyramine and dopamine administration (results not shown). All the above observations indicate that the monoamines behave synergistically with  $\text{Ca}^{2+}$  plus phosphate to amplify the induction of MPT, and that these effects are likely consequent to oxidative stress. The inhibitor of the monoamine oxidase (MAO) activity, toloxatone (TXT), as well as catalase, prevents the amplification of the swelling induced by serotonin, while it is ineffective on the swelling induced by  $\text{Ca}^{2+}$  alone (Fig. 3). Thus, the results obtained with TXT and catalase indicate that the observed effects of monoamines in amplifying the swelling of RLM are due to an oxidative stress caused by MAO-mediated oxidation products. The impression that the monoamines induce an oxidative stress are supported

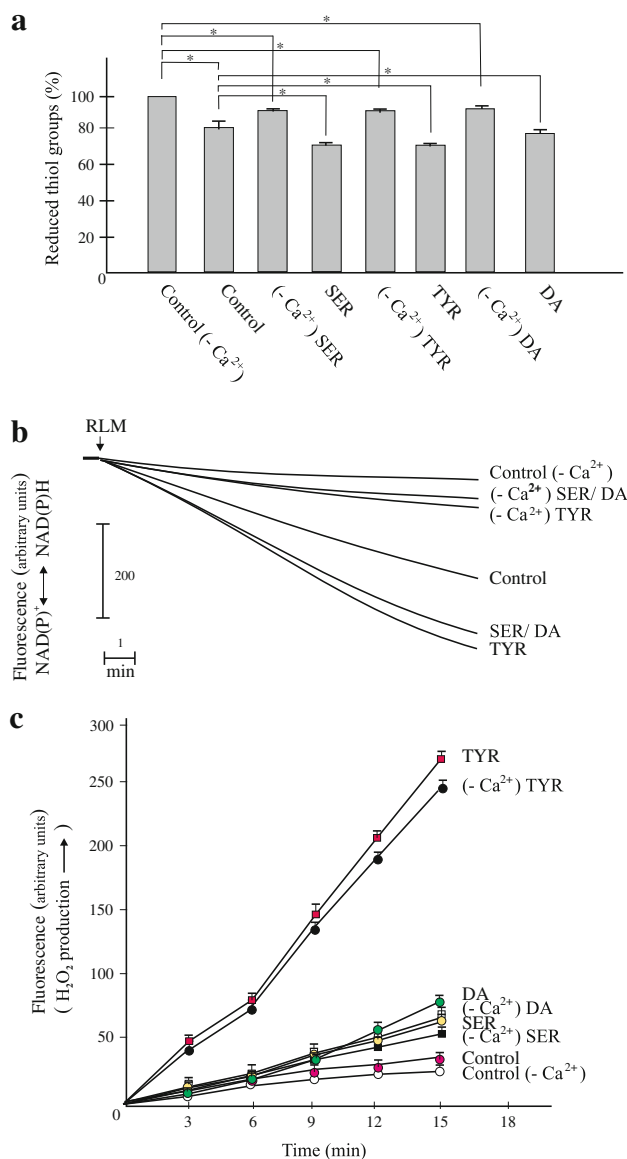


**Fig. 2** Effect of MPT inhibitors on mitochondrial swelling (a) and  $\Delta\Psi$  collapse (b) induced by  $\text{Ca}^{2+}$  plus serotonin. RLM were incubated in the standard conditions indicated in “Materials and methods”. Serotonin (SER) concentration was  $100\ \mu\text{M}$ , while:  $1\ \mu\text{M}$  CsA,  $500\ \mu\text{M}$  ADP,  $10\ \mu\text{M}$  NEM,  $5\ \mu\text{M}$  BKA,  $25\ \mu\text{M}$  BHT,  $3\ \text{mM}$  DTE or  $200\ \mu\text{M}$  DTNB were present when indicated. Traces are representative of six separate experiments



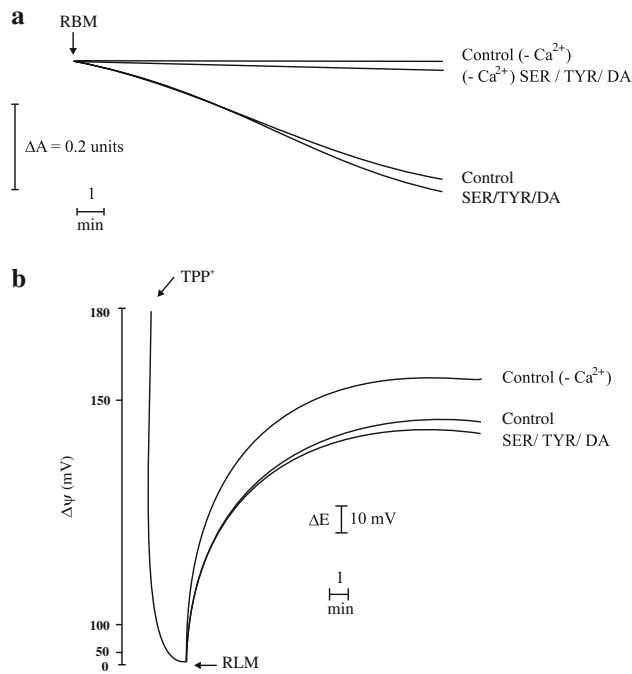
**Fig. 3** Effect of MAO inhibitor and catalase on swelling of RLM induced by  $\text{Ca}^{2+}$  plus serotonin. Where indicated  $50\ \mu\text{M}$   $\text{Ca}^{2+}$  and  $100\ \mu\text{M}$  SER were present. Toloxtatone (TXT) and catalase were added at concentrations of  $100\ \mu\text{M}$  and  $1,000\ \text{U}$ , respectively. Downward deflections indicate mitochondrial swelling. Traces are representative of four separate experiments

by the results of Fig. 4 showing that, besides  $\text{Ca}^{2+}$  (see Control), serotonin, dopamine and tyramine by themselves are able to decrease the content of mitochondrial sulfhydryl groups, although with a lower effectiveness than the cation (Fig. 4a). This level of the SH content is further decreased



**Fig. 4** Effects of calcium and monoamines on the redox state of sulfhydryl groups (a), and pyridine nucleotides (b), and hydrogen peroxide production (c) in RLM. Incubation conditions as described in “Materials and methods” with 50  $\mu\text{M}$   $\text{Ca}^{2+}$  and 100  $\mu\text{M}$  monoamines present where indicated. The levels of sulfhydryl groups were measured after 12 min incubation and their total content was considered as 100%. Values are means of four experiments. \* $p < 0.05$

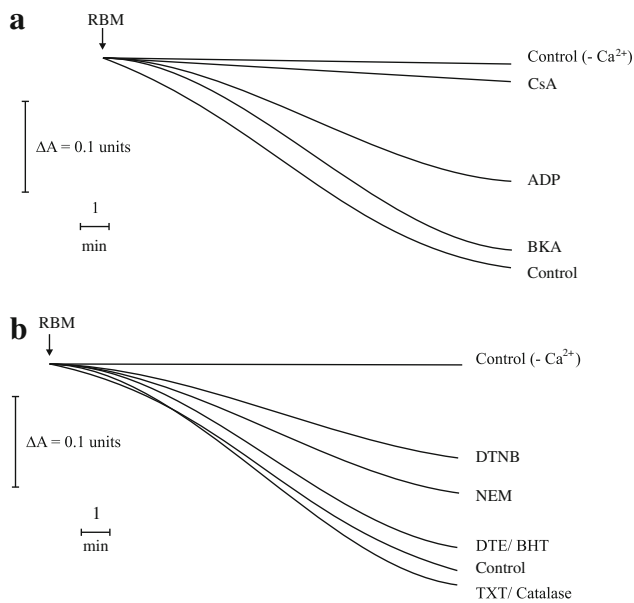
by incubation of the monoamines in the presence of  $\text{Ca}^{2+}$  is in accordance with opening of the PTP (Fig. 1a). To further strengthen this concept, similar results are obtained by detecting the pyridine nucleotide oxidation (Fig. 4b). This figure shows that  $\text{Ca}^{2+}$  alone induces a significant oxidation of these nucleotides, while monoamines alone do not. When calcium and monoamines are administered together the oxidation is strongly enhanced. Figure 4c shows that tyramine produces higher levels of  $\text{H}_2\text{O}_2$  than dopamine and serotonin, while  $\text{Ca}^{2+}$  generates a negligible amount of



**Fig. 5** Effect of monoamines on mitochondrial swelling (a) and  $\Delta\Psi$  drop (b) induced by  $\text{Ca}^{2+}$  in RBM. RBM were incubated in standard medium as described in “Materials and methods”. 100  $\mu\text{M}$   $\text{Ca}^{2+}$  was always present except when otherwise indicated; when reported the concentration of added monoamines was 100  $\mu\text{M}$ . Downward deflections indicate mitochondrial swelling (a). 2  $\mu\text{M}$   $\text{TPP}^+$  was used for  $\Delta\Psi$  measurements.  $\Delta E$  is the electrode potential (b). Traces are representative of six separate experiments

this peroxide. Contrary to the above data,  $\text{Ca}^{2+}$  and monoamines incubated together do not demonstrate marked synergistic increases in  $\text{H}_2\text{O}_2$  production relative to that produced by monoamines alone.

RBM incubated with phosphate and 50  $\mu\text{M}$   $\text{Ca}^{2+}$ , which is the ion concentration used for experiments performed with RLM, undergo less colloid-osmotic swelling, therefore 100  $\mu\text{M}$   $\text{Ca}^{2+}$  was generally used in the experiments with this type of mitochondria. RBM, energized by succinate plus rotenone and incubated with 100  $\mu\text{M}$   $\text{Ca}^{2+}$ , undergo a less dramatic swelling than that observed in RLM (Fig. 5a). In addition, the presence of serotonin, dopamine or tyramine does not induce appreciable changes in the absorbance of the mitochondrial suspension (Fig. 5a). RBM, in control conditions, exhibit a  $\Delta\Psi$  value of about 160 mV. When  $\text{Ca}^{2+}$  together with phosphate are present, a slight decrease of  $\Delta\Psi$  is observed, while the monoamines, as in the swelling experiments, do not display any significant effect (Fig. 5b). The analysis of effects of MTP inhibitors shows that CsA almost completely blocks the swelling induced by  $\text{Ca}^{2+}$  in RBM, while ADP and BKA exhibit a partial and negligible protection, respectively (Fig. 6a). The antioxidant agents DTE and BHT, as well as TXT and catalase, are almost, or completely,

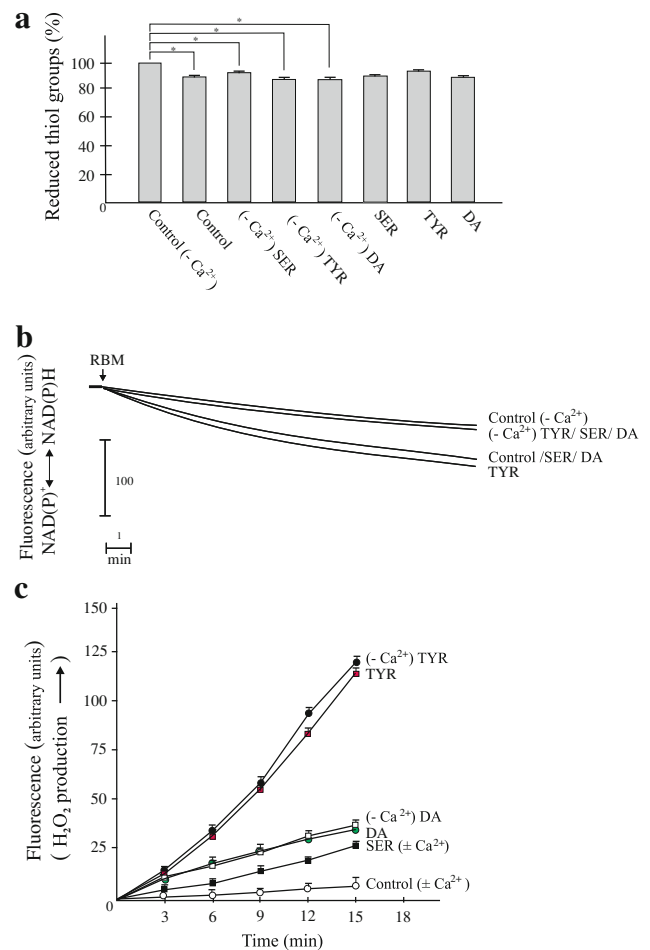


**Fig. 6** Effect of MPT inhibitors on mitochondrial swelling induced by  $\text{Ca}^{2+}$ . RBM were incubated in standard medium. When reported: 100  $\mu\text{M}$   $\text{Ca}^{2+}$ , 1  $\mu\text{M}$  CsA, 500  $\mu\text{M}$  ADP, 5  $\mu\text{M}$  BKA, 10  $\mu\text{M}$  NEM, 200  $\mu\text{M}$  DTNB, 25  $\mu\text{M}$  BHT, 3 mM DTE, 100  $\mu\text{M}$  TXT or 1000 U catalase were present. Traces are representative of six separate experiments

ineffective in inhibiting the swelling induced by  $\text{Ca}^{2+}$ , whereas the SH-reagents, DTNB and NEM, are partial inhibitors of this process (Fig. 6b). The effect of  $\text{Ca}^{2+}$  and monoamines on the levels of reduced sulfhydryl groups, is reported in Fig. 7a which shows that  $\text{Ca}^{2+}$  and all the tested monoamines, incubated by themselves, induce a decrease of the SH content of about 10%. In addition, unlike RLM, when the monoamines are incubated in RBM in the presence of  $\text{Ca}^{2+}$ , no further decrease in SH levels is found (Fig. 7a). Also in this case,  $\text{Ca}^{2+}$  alone induces some oxidation of pyridine nucleotides, while monoamines are not effective (Fig. 7b). The oxidation of the reduced pyridine nucleotides by  $\text{Ca}^{2+}$  is not increased when the cation is incubated together the monoamines (Fig. 7b). The production of  $\text{H}_2\text{O}_2$  by the monoamines in RBM is similar to that of RLM, while  $\text{Ca}^{2+}$  is completely ineffective in this type of mitochondria (Fig. 7c).

## Discussion

Several studies regarding the determination of the molecular mechanism of MPT induction have been performed in RLM. In particular, this type of mitochondria has been used as a target where the biogenic amines behaved as inhibitors or inducers of the above phenomenon. These studies concerned principally the action of the natural polyamines (Sava et al. 2006; Toninello et al. 2004), but



**Fig. 7** Effects of calcium and monoamines on the redox state of sulfhydryl groups (a), pyridine nucleotides (b), and hydrogen peroxide production (c) in RBM. Incubation conditions and monoamines concentrations as in Fig. 4 except that 100  $\mu\text{M}$   $\text{Ca}^{2+}$  was added. Total content of reduced sulfhydryl groups were considered as 100%. Values are means of four experiments. \* $p < 0.05$

also that of the propargylamines (Marcocci et al. 2002) and diamines, in particular agmatine (Battaglia et al. 2007). As far as the monoamines are concerned, only tyramine and dopamine have been investigated as MPT inducers in RLM (Marcocci et al. 2002; Brenner-Lavie et al. 2008).

The present study deals with the different effects exhibited by the monoamines serotonin, tyramine, and dopamine on the phenomenon of  $\text{Ca}^{2+}$ -dependent MPT induction in RLM and RBM. As shown in the experiments of Figs. 1 and 2, the monoamines are able to significantly amplify this phenomenon in RLM, through an oxidative stress, the process also triggered by  $\text{Ca}^{2+}$ . The order of effectiveness in inducing mitochondrial swelling and  $\Delta\Psi$  collapse in this model is serotonin > tyramine > dopamine. We show that monoamines amplify the PTP opening in RLM by the observation that typical inhibitors of this phenomenon, such as CsA, ADP, and BKA, completely

prevent it (Fig. 2a, b). This phenomenon is attributed to cellular oxidative stress and supported by the observation that the antioxidant agents DTE and BHT, as well as the SH-reagent DTNB and the alkylating agent NEM, significantly inhibit this process (Fig. 2a, b).

It is well known that a mitochondrial MAO, located on the external side of outer membrane, releases hydrogen peroxide as a by-product of monoamine oxidation. The observation that both TXT and catalase partially inhibit the RLM swelling (Fig. 3) suggests that the swelling amplification induced by serotonin is due to the oxidative stress caused by MAO-mediated generation of  $H_2O_2$ . This is also supported by the fact that TXT and catalase reduce the swelling to almost the same extent induced by  $Ca^{2+}$  alone. A further confirmation that the opening and the amplification of PTP is dependent on oxidative stress is illustrated in Fig. 4, where the monoamines or  $Ca^{2+}$  can each induce the oxidation of sulfhydryl groups and pyridine nucleotides in parallel with  $H_2O_2$  generation. The lack of correlation between the tyramine-produced high amounts of  $H_2O_2$ , and oxidation of sulfhydryl groups and pyridine nucleotides might be due to the fact that the  $H_2O_2$  produced exceeds the saturation levels of the oxidation systems. The  $Ca^{2+}$ -induced oxidation of thiols and pyridine nucleotides is most likely due to the interaction of the cation with the membrane cardiolipins that causes a disorganization of the membrane and consequent alteration of ubiquinone mobility (Grijalba et al. 1999). This would favour the formation of the semiquinone radical and, consequently, of superoxide anion and hydrogen peroxide (Agostinelli et al. 2004). However, the oxidation of thiols and pyridine nucleotides induced by the monoamines is likely due to the generation of  $H_2O_2$  by MAO activity (Fig. 4).

It is generally accepted that the opening of PTP requires the interaction of  $Ca^{2+}$  with a specific site located in the AdNT (Halestrap and Brenner 2003) or, as recently proposed, in the phosphate carrier (Leung et al. 2008), with the oxidation of two specific cysteine SH groups located in these carriers. As the presence of  $Ca^{2+}$  is necessary we conclude that monoamines alone are not able to open the PTP. However, they potentiate the effect of  $Ca^{2+}$  in RLM by means of further production of  $H_2O_2$  as the result of an increased MAO activity.

In RBM, MPT is induced by  $Ca^{2+}$  by a mechanism apparently similar to that proposed for RLM. In fact, in RBM,  $Ca^{2+}$  induces mitochondrial swelling and  $\Delta\Psi$  drop, even if to a lesser extent than that observed in RLM (Figs. 1, 5). Moreover, the well-established inhibitor CsA also prevents this process in this mitochondrial type (Figs. 5, 6a). However, the monoamines, under our experimental conditions, do not cause significant changes in the swelling and  $\Delta\Psi$  drop induced by  $Ca^{2+}$  (Fig. 5). Furthermore, apart from CsA, all the other inhibitors were

unable to prevent the  $Ca^{2+}$ -induced PTP. In fact, BKA, DTE and BHT were completely ineffective, while ADP, DTNB and NEM exhibited only partial protection (Fig. 6). This would mean that the classic mechanism proposed for RLM is not suitable for RBM. The observation that CsA completely blocks the PTP opening confirms the involvement of cyclophilin in this process in both RLM and RBM, but the ineffectiveness exhibited by ADP and BKA suggests the involvement of a protein other than AdNT. Indeed, the partial or complete lack of protection by the antioxidant agents, as well as the ineffectiveness exhibited by the monoamines, suggests that in RBM the opening of the PTP is not necessary responsive to oxidative stress induced by hydrogen peroxide. In this regard the slight decrease in SH content induced by  $Ca^{2+}$  and monoamines themselves does not influence MPT induction and does not appear additive in RBM (Fig. 7a). It is also noteworthy that the thiol oxidation by the monoamines is not accompanied by a parallel oxidation of pyridine nucleotides (Fig. 7b), suggesting that the oxidation of the above compounds is not correlated with one another. Another observation is that the monoamines are able to produce a consistent amount of  $H_2O_2$  far higher than that produced by  $Ca^{2+}$ , and the presence of calcium does not significantly change the  $H_2O_2$  generation by monoamines (Fig. 7c). Most likely, the observed slight oxidation of thiols and pyridine nucleotides in the presence of  $Ca^{2+}$  consumes the  $H_2O_2$  produced in this condition leaving almost undetectable available amounts of this compound. In conclusion, the results here reported demonstrate a significant difference between RLM and RBM in the mechanism of MPT induction. RBM do not require  $H_2O_2$ -dependent oxidative stress to undergo this process, as observed in RLM. This statement challenges the general opinion concerning the mechanism triggered by  $Ca^{2+}$  in the presence of phosphate. Most likely in RBM,  $H_2O_2$ , indirectly produced by  $Ca^{2+}$ , or by monoamine oxidation, is able to oxidize specific sulfhydryl groups (see Fig. 7a), but not the critical thiols that take part in the opening of PTP. The different behaviour of the two types of mitochondria may be related to the tissue-specific neuro-physiological processes and adaptation of the metabolic networks to the different tissue functions. It is important to mention that neurons produce monoamines while mature hepatocytes do not. However, under pro-inflammatory or stress situations monoamines can be produced by immune cells infiltrated in liver. The different expression/properties of the MAO isoenzymes between liver and brain (Richardson 1993, Remaury et al. 1999, Youdim et al. 2001) (may be with different protein-protein interaction characteristics with PTP elements) could be on the bases of the observed different actions. So even if it is premature to draw definitive conclusions, it can be possible to hypothesize that the here reported monoamine roles

could be correlated with monoamine and MAO-dependent neuropathologies such as Parkinson's disease (Richardson 1993, Remaury et al. 1999; Youdim et al. 2001, Rajput et al. 2008) and liver pathologies such as cirrhosis and steatohepatitis (Butterworth 2000, Nocito et al. 2007).

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**Conflict of interest** The authors declare that they have not conflict of interest.

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