ORIGINAL ARTICLE

Agmatine prevents the Ca^{2+}-dependent induction of permeability transition in rat brain mitochondria

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Abstract The arginine metabolite agmatine is able to protect brain mitochondria against the drop in energy capacity by the Ca^{2+}-dependent induction of permeability transition (MPT) in rat brain mitochondria. At normal levels, the amine maintains the respiratory control index and ADP/O ratio and prevents mitochondrial colloid-osmotic swelling and any electrical potential ($\Delta\Psi$) drop. MPT is due to oxidative stress induced by the interaction of Ca^{2+} with the mitochondrial membrane, leading to the production of hydrogen peroxide and, subsequently, other reactive oxygen species (ROS) such as hydroxyl radicals. This production of ROS induces oxidation of sulfhydryl groups, in particular those of two critical cysteines, most probably located on adenine nucleotide translocase, and also oxidation of pyridine nucleotides, resulting in transition pore opening. The protective effect of agmatine is attributable to a scavenging effect on the most toxic ROS, i.e., the hydroxyl radical, thus preventing oxidative stress and consequent bioenergetic collapse.

Abbreviations

ADC	Arginine decarboxylase
AGM	Agmatine
BKA	Bongkrekic acid
CsA	Cyclosporin A
$\Delta\Psi$	Electrical transmembrane potential
$\Delta\mu_{H^+}$	Transmembrane electrochemical gradient
DMO	5,5′-dimethyl-oxazolidine-2,4-dione
DTE	Dithioerythritol
MPT	Mitochondrial permeability transition
NEM	N-Ethylmaleimide
NOS	Nitric oxide-synthase
ODC	Ornithine decarboxylase
RBM	Rat brain mitochondria
RCI	Respiratory control index
RKM	Rat kidney mitochondria
RLM	Rat liver mitochondria
ROS	Reactive oxygen species
SSAT	Spermidine/spermine-N^1-acetyltransferase
TPP^+	Tetraphenylphosphonium

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Introduction

The dicationic biogenic amine agmatine, derived from decarboxylation of arginine by the activity of arginine decarboxylase (ADC), can induce a large number of significant physiological effects (Raasch et al. 2001; Grillo and Colombatto 2004). In particular, agmatine hinders

activation of the proliferative pathway triggered by arginine and inhibits the activity of NO-synthase (NOS), thus evidencing the primary role it plays in these pathways (Satriano 2004). Agmatine suppresses cell growth by reducing intracellular polyamine levels with the induction of the polyamine autoregulatory protein antizyme that inhibits ornithine decarboxylase (ODC), the first enzyme of the polyamine biosynthetic pathway (Higashi et al. 2004; Satriano et al. 1998). Agmatine reduces polyamine content also by inducing spermidine/spermine-N^1-acetyltransferase (SSAT) (Vargiu et al. 1999). It is noteworthy that agmatine is transported in mammalian cells by means of the polyamine transport system, but polyamine transport activity is correlated with proliferation rate, whereas agmatine targets cells with high proliferative kinetics to suppress their growth (Isome et al. 2007). The mechanism involved in growth suppression by agmatine is not fully understood. Several studies regarding antigrowth effects take into account the induction of apoptosis and agmatine, by inducing depletion of polyamine level, a common and potentially causal factor of apoptosis, considered as an antigrowth agent. Apoptosis is a process of programmed cell death occurring during normal cell life, which takes place as a balancing mechanism toward cellular proliferation in maintaining cell population. This process is energy-dependent and also occurs during disease states in counteracting neoplastic proliferation, thus constituting not only an important defense mechanism against tumor development but also a host defense against pathogenic agents. Apoptosis may be triggered by two general pathways: (1) the extrinsic or death receptor pathway, and (2) the intrinsic or mitochondrial pathway. These pathways may have a reciprocal influence and both act at the level of execution caspase, i.e., caspase 3. The activation of caspase 3 triggers the caspase cascade, leading to apoptotic events including DNA fragmentation and apoptotic body formation. The intrinsic pathway is induced by alterations of the mitochondrial membrane, leading to the phenomenon of mitochondrial permeability transition (MPT) (for a review on MPT, see Zoratti and Szabò 1995) and the opening of the transition pore. In several cases, the induction of MPT may require the oxidation of critical thiol groups (McStay et al. 2002), as the result of oxidative stress in which biogenic amines play a prominent role (Agostinelli et al. 2004). This phenomenon provokes bioenergetic collapse, with the complete drop of solute gradients, colloid-osmotic swelling of the mitochondrial matrix, outer membrane rupture, and release of pro-apoptotic factors cytochrome c, AIF, Smac/DIABLO, etc. Thus, these factors activate caspase 3 and subsequent events. As has been previously reported, agmatine is transported in liver (Salvi et al. 2006) and brain mitochondria (Battaglia et al. 2009), and demonstrates different behaviour among them at the level of MPT induction. Agmatine, at 10 or 100 μM concentrations, can induce oxidative stress in rat liver mitochondria (RLM), most probably by the production of H_2O_2 and other reactive oxygen species (ROS), in a reaction catalyzed by a mitochondrial amine oxidase. This oxidative stress causes loss of the bioenergetic capacity of mitochondria and amplification of the MPT induced by supraphysiological Ca^{2+} concentrations. At higher concentrations (0.5–1 mM), agmatine generates larger amounts of H_2O_2 than the redox level, although it maintains the normal level of ATP synthesis and prevents MPT induction by Ca^{2+}. This protective effect of high agmatine concentration has been proposed to be due to a self-scavenging action against ROS produced by itself and Ca^{2+}, exhibited by the amount of the amine not yet oxidized by amine oxidase. A very recent paper also reports that, in response to Ca^{2+}-induced oxidative stress in rat kidney mitochondria (RKM), agmatine behaves like a free radical scavenger by preventing ROS generation. The functional consequence is a protective effect against Ca^{2+}-induced MPT (Arndt et al. 2009). In considering the recent discovery of a transport system for agmatine in rat brain mitochondria (RBM), having different characteristics from that present in RLM, this study evaluates the effects of agmatine in RBM at the level of bioenergetic functions and MPT, in order to examine the potential regulatory effect of this amine on mitochondrial energy capacity and eventually on apoptosis.

Materials and methods

Isolation of rat brain mitochondria (RBM)

Rat brain mitochondria were isolated by conventional differential centrifugation method and purified by the Ficoll gradient method, according to Nicholls (1978), 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; 0.3% BSA was added during homogenization and the first step of purification) 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 plus 1 mM ATP and layered on top of a discontinuous gradient, composed of 2 ml of isolation medium containing 12% (w/v) Ficoll, 3 ml of isolation medium containing 9% (w/v) Ficoll, and 3 ml of isolation medium containing 6% (w/v) Ficoll. The gradient was 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 again suspended 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 were approved by the Italian Ministry of Health.

Standard incubation procedures for RBM

Rat brain mitochondria (1 mg protein/ml) were incubated in a water-jacketed cell at 20°C. The standard medium contained 200 mM sucrose, 10 mM HEPES (pH 7.4), 5 mM succinate, 1 mM phosphate and 1.25 µM rotenone. Variations and/or other additions are described with the individual experiments presented.

Determination of mitochondrial function

Electrical transmembrane potential ($\Delta\Psi$) was calculated by determining the distribution of the lipid soluble cation tetraphenylphosphonium (TPP^+) through the inner membrane, measured by a TPP^+ selective electrode prepared in our laboratory, according to previously published procedures (Kamo et al. 1979). ΔpH was measured from the distribution of DMO ([^{14}C] 5,5'-dimethyl-oxazolidine-2,4-dione) through the inner membrane (Rottenberg 1979). Mitochondrial matrix volume was determined from the uptake of [^{14}C]sucrose and ^3H_2O according to the method of Palmieri and Klingenberg (1979). Oxygen uptake was measured by a Clark electrode. Respiratory control index and P/O ratio were calculated as reported previously (where P/O is the ratio of the number of ATP molecules formed to the number of oxygen molecules reduced by electron transport) (Battaglia et al. 2005).

Determination of mitochondrial swelling

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

Measurement of oxidative status of RBM

The protein sulfhydryl group oxidation assay was performed as in Santos et al. (1998). The redox level of glutathione was monitored as described in Tietze (1969). The redox state of endogenous pyridine nucleotides was followed fluorometrically in an Aminco-Bowman 4-8202 spectrofluorometer with excitation at 354 nm and emission at 462 nm. The production of H_2O_2 in mitochondria was measured fluorometrically by the Scopoletin method (Loschen et al. 1973) in an Aminco-Bowman 4-8202 spectrofluorometer.

Results

Mitochondria functions are closely connected with the integrity of the mitochondrial membrane, which provides a correct electron flux along the respiratory complexes, essential to establish the electrochemical gradient ($\Delta\mu_{H^+}$), the driving force for ATP synthesis in energy-transducing membranes. The experiments reported in this section were performed with the aim of evaluating the effect of agmatine on bioenergetic parameters in the absence and presence of Ca^{2+}. As shown in Fig. 1, agmatine, at 10 or 100 µM concentrations, does not reveal significant changes in the $\Delta\Psi$ value of RBM incubated in standard medium. However, 1 mM agmatine induces a considerable drop in $\Delta\Psi$ of about 20 mV, although this effect is not attributable to a damaging effect of mitochondrial membrane, but is due to the electrophoretic accumulation of agmatine in the mitochondrial matrix. In fact, the entry of positive charges of agmatine in mitochondria neutralizes the inner negative potential, by compelling them to further ejection of protons with a corresponding increase in ΔpH, while $\Delta\mu_{H^+}$ remains constant. The inset in Fig. 1 shows the increase in the 58 ΔpH value by about 20 mV in the presence of agmatine.

Other parameters linked to the energy capacity of mitochondria are the respiratory control index (RCI) and the ADP/O ratio. Respiratory control index is the ratio between the respiratory rate of mitochondria in resting conditions (no ATP synthesis) and the same rate during ATP synthesis. ADP/O ratio is the ratio between n moles of synthesized ATP and atoms of consumed oxygen. The values of both parameters highlight coupling between the activities of ATP-synthase and the respiratory chain.
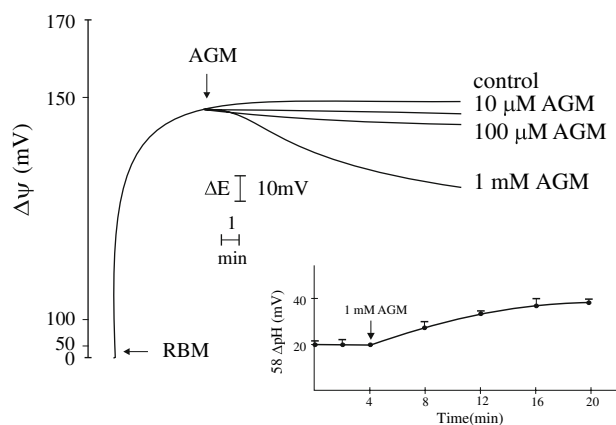

Fig. 1 Effect of agmatine on membrane potential of RBM. RBM were incubated in standard medium, as described in "Materials and methods". Agmatine (AGM) concentrations are indicated at side of traces. 2 µM TPP^+ was present for $\Delta\Psi$ measurements. ΔE electrode potential. *Inset* effect of 1 mM agmatine on ΔpH. RBM were incubated in standard medium in presence of 400 µM [^{14}C]DMO (1 µCi/mmol), 5 mM [^3H]glycerol (100 µCi/mmol). Five additional experiments exhibited the same trend

Experimental results are listed in Table 1 and show that agmatine, in the absence of Ca^{2+}, does not affect the energy parameters; at 1 mM concentration it even increases them. Instead, in the presence of 30 μM Ca^{2+}, RCI and P/O are significantly lowered, if compared with the control, while, in the presence of agmatine, the damaging effect is prevented in a dose-dependent fashion. The mitochondrial respiratory chain is well known to be the main producer of hydrogen peroxide in the cell. In normal conditions, the safety systems present in mitochondria, i.e., superoxide dismutase, catalase, and the glutathione peroxidase/glutathione reductase system, are able to eliminate most of the produced hydrogen peroxide. However, some ROS may escape this protective effect, thus accounting for the fact that they can be measured in resting conditions. In this regard, the results shown in Fig. 2a show that RBM incubated in standard medium produces about 0.6 nmol/mg prot of H_2O_2. The presence of agmatine at 10, 100 and 1,000 μM concentrations reduces this amount, showing dose-dependent behavior. The same concentrations of agmatine do not exhibit any appreciable effect in the redox state of sulfhydryl groups (Fig. 2b) or pyridine nucleotides (Fig. 2c).

These results show that agmatine, although it produces H_2O_2, is ineffective in inducing any oxidant effect at mitochondrial level. As mentioned above, when mitochondria are incubated in the presence of supraphysiological Ca^{2+}, the organelles undergo the phenomenon of MPT. This is the case of the results shown in Fig. 3, demonstrating that RBM, in the presence of 30 μM Ca^{2+}, exhibit colloid-osmotic swelling, revealed by the apparent decrease in the absorbance of mitochondrial suspension by about 0.15 units (panel a) and the collapse in $\Delta\Psi$ (panel b). The presence of the immunosuppressant cyclosporin A (CsA) completely abolishes mitochondrial swelling, as well as other effectors such as bongkrekic acid (BKA), dithioerythritol (DTE), and N-ethylmaleimide (NEM). Agmatine administration also prevents both mitochondrial swelling and $\Delta\Psi$ drop, again in a dose-dependent manner, even at low concentrations (e.g., 10–100 μM), i.e., in

Table 1 Respiratory parameters of RBM in presence of agmatine

Condition	RCI	ADP/O
Control	4.3	1.38
10 μM AGM	4.5	1.42
100 μM AGM	3.9	1.36
1 mM AGM	5.1	1.33
Ca^{2+}	1.5 ± 0.03	1.05
Ca^{2+} + 10 μM AGM	2.4 ± 0.01	1.15
Ca^{2+} + 100 μM AGM	3.6 ± 0.02	1.28
Ca^{2+} + 1 mM AGM	4.5 ± 0.01	1.40

conditions able to induce MPT in RLM. The opening of the transition pore induced by Ca^{2+} is the result of indirect oxidative stress provoked by the cation, as confirmed by the results of Fig. 4a, which shows that Ca^{2+} increases H_2O_2 generation when compared with the resting condition shown in Fig. 2a. Depending on its concentration, agmatine significantly reduces this amount of H_2O_2. The production of hydrogen peroxide in the presence of Ca^{2+} induces oxidative stress in mitochondria, detectable by increased oxidation of sulfhydryl groups (Fig. 4b) and pyridine nucleotides (Fig. 4c). In evaluating these effects caused by Ca^{2+} transport, agmatine protects the oxidation of both sulfhydryl groups (Fig. 4b) and pyridine nucleotides (Fig. 4c) always in a dose-dependent manner.

As emphasized in a previous paper (Battaglia et al. 2009), the agmatine concentrations used in this study are higher than those normally present in body fluids and in the cytosol. However, it should be noted that the suspension in the medium of isolated mitochondria has a density far higher than that of mitochondria in the cell, so that, in order to obtain comparable results, it is necessary to use higher agmatine concentrations.

Discussion

The bioenergetic functions of agmatine at mitochondrial level have previously been studied both in RLM (Salvi et al. 2006) and RKM (Arndt et al. 2009), in order to ascertain whether this amine can influence the energy capacity of mitochondria and their peculiar membrane properties, in particular at the level of transition pore opening. The observation that agmatine has been found in neuronal mitochondria (Gorbatjuk et al. 2001) together with its metabolic enzymes, arginine decarboxylase and agmatinase, and the imidazoline receptor I_2 (Regunathan and Reis 2000; Horyn et al. 2005; Sastre et al. 1996), when viewed together with the very recent discovery of a transport system for this amine in RBM (Battaglia et al. 2009), has aroused interest in evaluating the possible effects of agmatine on mitochondrial bioenergetic functions, also in this type of mitochondria. The most important implications of agmatine, at the brain mitochondria level, reveal a general protective effect exhibited on the main bioenergetic parameters and, in particular, against MPT induction (see Table 1; Fig. 3). Agmatine maintains the normal values of the RCI and ADP/O ratio, affected by the presence of Ca^{2+}, thus demonstrating the capacity to preserve close coupling between ATP synthesis and oxygen uptake, the peculiar characteristic of the chemiosmotic theory. It is noteworthy that agmatine, in RBM, does not exhibit any change in membrane impermeability (Fig. 1), as well as any oxidative stress, when incubated alone

Fig. 2 Dose-dependent effect of agmatine on hydrogen peroxide production (**a**), redox state of sulfhydryl groups (**b**), and pyridine nucleotides (**c**). Incubation conditions and agmatine concentrations as in Fig. 1. Mean values ± SD of five experiments are reported for **a** and **b**. **c** A typical experiment; three others gave identical results
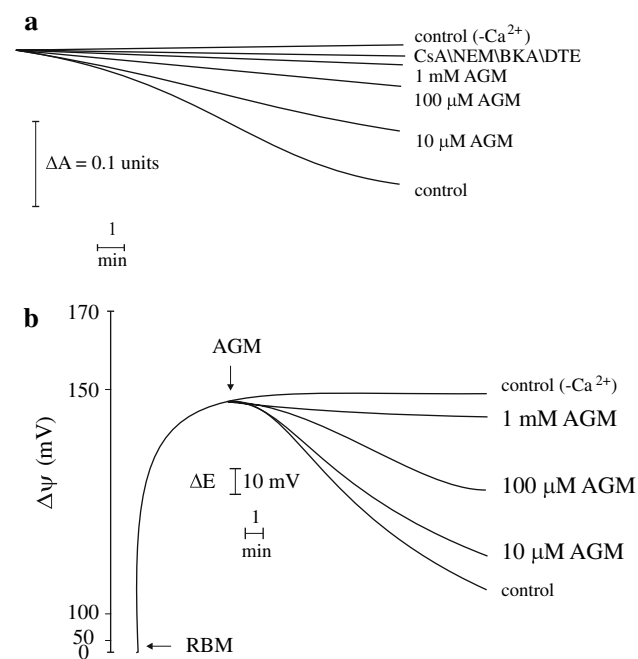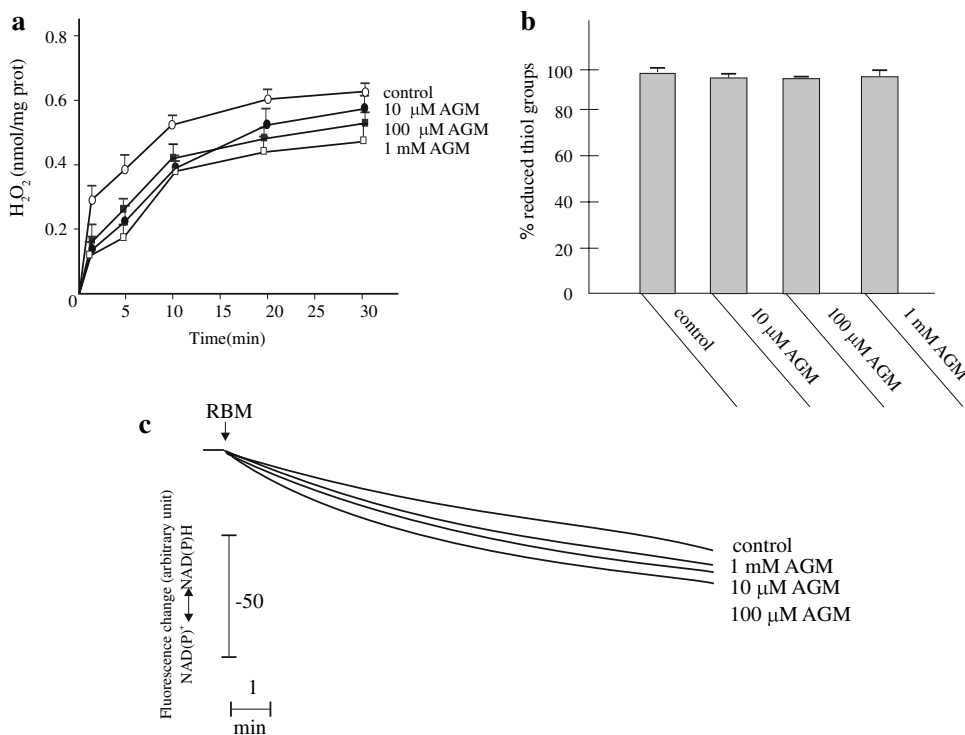
Fig. 3 Effect of agmatine on mitochondrial swelling induced by Ca^{2+}. RBM were incubated in standard medium in presence of 30 µM Ca^{2+}. Agmatine (AGM) concentrations are indicated at side of traces, where present 1 µM CsA, 10 µM NEM, 5 µM BKA or 1 mM DTE. Five other experiments gave superimposable curves

(Fig. 2). The protection exhibited by agmatine on MPT induction by Ca^{2+} (Fig. 3) highlights another significant biological effect of this amine in protecting RBM against bioenergetic collapse and the redox catastrophe (Fig. 4) caused by this process, and its potential implication in the regulation of apoptosis. The mechanism for this protection has not been completely demonstrated but, taking into account previous observations regarding the effects of agmatine and polyamines in RLM (Salvi et al. 2006; Sava et al. 2006), it is possible that agmatine acts as a scavenger against ROS produced by the interaction of Ca^{2+} with the mitochondrial membrane. This mechanism, in the view of Casero's group regarding the polyamine spermine (Ha et al. 1998; Rider et al. 2007), proposes that agmatine reacts with hydroxyl radical, most probably produced by the interaction of H_2O_2 with some iron of the Fe–S centers present in respiratory complexes (Battaglia et al. 2007) to form dihydroxyaminobutyl-guanidine. Then, by spontaneous dehydration and subsequent hydrolysis, guanidobutyric aldehyde is formed. This characteristic of agmatine would explain its preventive effect against MPT induced by Ca^{2+}, most probably due to the activity of the hydroxyl radical. It should be emphasized that the scavenging effect demonstrated by polyamines is effective only against the hydroxyl radical (Sava et al. 2006; Ha et al. 1998). These results, obtained in RBM, show substantial differences when compared with those in RLM (Battaglia et al. 2007). In the latter case, as mentioned above, agmatine at low concentrations induces MPT. This difference is due to the presence in RLM of an amino oxidase which oxidizes agmatine by producing H_2O_2 and, most probably, OH^- (Cardillo et al. 2009). RBM do not contain this enzyme, so that there

Fig. 4 Dose-dependent effect of agmatine on hydrogen peroxide production (a), redox state of sulfhydryl groups (b), and pyridine nucleotides (c) in presence of Ca^{2+}. Incubation conditions and agmatine concentrations as in Fig. 3. Mean values ± SD of five experiments are reported for a and b. c A typical experiment; three others gave identical results
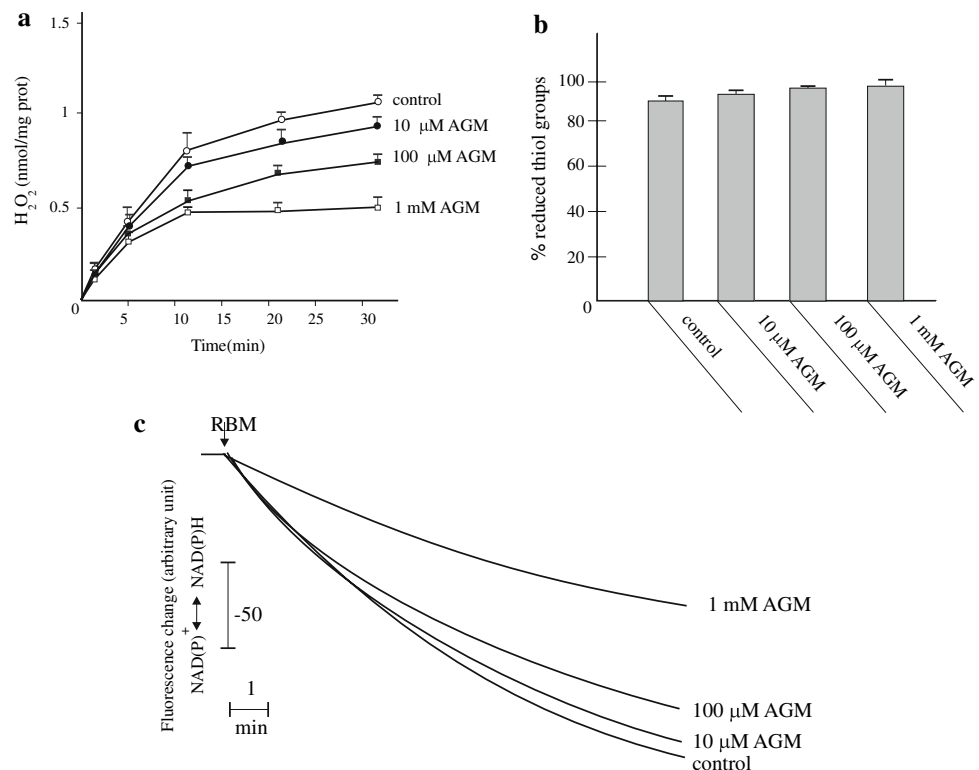

is no consistent production of ROS and, consequently, no induction of MPT by agmatine itself. Thus, the amine, even at low concentrations, exhibits the above-described scavenging effect. These results show that, when MPT is induced by Ca^{2+}, the event which most probably triggers the process is the production of ROS, which occurs by the interaction of Ca^{2+} with the respiratory chain (McStay et al. 2002). However, it should be noted that the phenomenon of MPT in RBM can also be induced by other mechanisms. A very recent paper reports that MPT may be induced by oligomeric BAX, without involving oxidative stress (Li et al. 2008). The presence of agmatine prevents the opening of the transition pore, induced by supraphysiological Ca^{2+} levels, to differing degrees of efficacy depending on concentration (Fig. 3). This observation flanks the protective effects of agmatine on respiratory parameters (Table 1), and means that it may be considered as a protective agent of mitochondrial bioenergetics.

In conclusion, this study gives rise to speculations on the effects of agmatine in vivo, as it may behave as a stabilizer of cell energy content.

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