UNIVERSITA' DEGLI STUDI DI PADOVA

Dipartimento di CHIMICA BIOLOGICA

SCUOLA DI DOTTORATO DI RICERCA IN: BIOCHIMICA E BIOTECNOLOGIE
INDIRIZZO: BIOCHIMICA E BIOFISICA
CICLO XXI

TESI DI DOTTORATO

BIOGENIC AMINES AS REGULATORS OF MITOCHONDRIAL FUNCTIONS: ROLES OF AGMATINE

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Table of contents

Abstract

Agmatine is a dicationic amine at physiological pH, formed by decarboxylation of arginine catalyzed by arginine decarboxylase. It acts on polyamine metabolism by inhibiting nitric oxyde synthase and activating spermidine/spermine acetyltransferase as well as the antizyme of ornithine decarboxylase. Agmatine is metabolized by agmatinase to form urea and putrescine, suggesting that it is a polyamine precursor. Agmatine is transported to organs by an energy-dependent mechanism, whereas increased cellular concentrations promotes apoptosis. Agmatine and its metabolic enzymes arginine decarboxylase and agmatinase have also been recognized in mitochondria, as well as imidazoline I_2 receptor. These observations and the results obtained in these years, during my work, evidence a close relationship between agmatine and mitochondria.

The aim of this work is to study the action of agmatine as regulator of mitochondrial functions, compared with the effect of polyamines (i.e. spermine), in isolated rat mitochondria from different organs: liver, brain and kidney.

The first part of the work focuses on agmatine uptake by mitochondria with characterization of the transport system. A comparison among the agmatine transport mechanism in mitochondria isolated from different organs is reported.

In the second part is reported the action of this amine on mitochondrial permeability transition induction, again with the above mentioned comparison but together the effects of spermine. The amine exhibits protective effects against the phenomenon in kidney and brain mitochondria, whereas in liver mitochondria it exhibits double behavior, that is, induction at low concentrations and protection at high concentrations. The possible explanation is the presence of a specific amino oxidase in liver mitochondria.

Finally, in the third part, the synthesis of agmatine using an alternative reaction to that of arginine decarboxylase is reported. The presence of an amidinotransferase reaction has been found in rat kidney mitochondria and in a proximal tubule cell line as the results of a first purification step. This observation could correlate the synthesis of agmatine with a regulation mechanism of polyamine concentration in cells.

In conclusions the results obtained with this study put in evidence agmatine as a physiological regulator of polyamine content in the cell, rather than a simple polyamine precursor, as proposed by some authors. Moreover, this investigation point out the important

physiological role of mitochondria activity as mediators of this process. Indeed, the action of this amine in mitochondrial permeability transition of isolated mitochondria explains its effect on cell proliferation and apoptosis.

Sommario

L'agmatina è un'amina è formata dalla decarbossilazione dell'arginina in una reazione catalizzata dall'arginina decarbossilasi ed è caratterizzata dalla presenza di due cariche a pH fisiologico. L'agmatina agisce sul metabolismo delle poliamine inibendo la ossido nitrico sintasi e attivando la spermidina/spermina acetiltransferasi e l'antizima dell'ornitina decarbossilasi. L'agmatina viene metabolizzata dall'agmatinasi formando urea e putrescina, suggerendo che sia un precursore delle poliamine. Viene trasportata agli organi da un meccanismo energia-dipendente e, un aumento della sua concentrazione, promuove l'induzione dell'apoptosi. L'agmatina e gli enzimi del suo metabolismo, arginina decarbossilasi e agmatinasi, così come i recettori imidazolinici I_2, ai quali l'amina si lega, sono stati ritrovati nei mitocondri. Queste osservazioni e i risultati ottenuti in questi anni, durante il mio lavoro, evidenziano una stretta relazione tra agmatina e mitocondri.

Lo scopo di questa ricerca è studiare l'azione dell'agmatina come regolatore delle funzioni mitocondriali e confrontarne gli effetti con quelli delle poliamine (ad esempio spermina), in mitocondri isolati da organi differenti di ratto: fegato, cervello e rene.

La prima parte del lavoro riporta il trasporto dell'agmatina all'interno dei mitocondri e la caratterizzazione del sistema di trasporto. Vengono comparati i meccanismi di trasporto dell'agmatina nei mitocondri isolati dai diversi organi.

Nella seconda parte viene riportata l'azione di quest'amina sull'induzione della transizione di permeabilità mitocondriale, di nuovo comparando gli effetti nei diversi organi ma anche con la spermina. L'amina ha un effetto protettivo contro il fenomeno in rene e cervello, mentre nel fegato il suo comportamento è duplice: induce a basse concentrazioni e protegge ad alte. Per spiegare tale differenza, si ipotizza la presenza di una specifica amino ossidasi nei mitocondri di fegato.

Infine, nella terza parte, viene studiata la sintesi di agmatina tramite una reazione alternativa all'arginina decarbossilasi. La presenza di una reazione amidinotransferasica è stata riscontrata in mitocondri di rene di ratto e in un linea cellulare di tubulo prossimale. Vengono riportati anche i risultati relativi ad un primo step di purificazione. Queste osservazioni potrebbero correlare la sintesi di agmatina con un meccanismo per la regolazione della concentrazione di poliamine nelle cellule.

In conclusione, i risultati ottenuti mettono in evidenza come l'agmatina agisca da regolatore fisiologico del contenuto di poliamine nella cellula piuttosto che comportarsi come un semplice precursore delle poliamine, come viene considerata da alcuni autori. Questa ricerca dimostra, inoltre, come i mitocondri siano importanti mediatori fisiologici di questo processo. Infatti, l'azione di quest'amina sulla transizione di permeabilità mitocondriale spiega i suoi effetti sulla proliferazione cellulare e sull'apoptosi.

Abbreviations

ADC	arginine decarboxylase
AdNT	adenine nucleotide translocase
AGM	agmatine
AIF	apoptosis inducing factors
AO-AGM	N-(3-aminooxypropyl)-guanidine
BHT	butyl-hydroxytoluene
BKA	bongkrekic acid
BSA	bovine serum albumine
CsA	cyclosporin A
CypD	cyclophylin D
DAO	diamine oxidase
DFMO	difluoromethylornithine
DTNB	5,5'-dithio-bis-2-nitrobenzoic acid
DTT	ditiothreitol
EMT	extraneuronal monoamine transporter
FCCP	carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone
GAPA	N-(3-aminopropoxy)guanidine
HRP	horseradish peroxidase
MAO	monoamine oxidase
MCT	mouse kidney proximal tubule cell line
MGBG	methylglyoxal bis(guanylhydrazone)
MPT	mitochondrial permeability transition
NEM	N-ethylmaleimide
NGPG	N-(3-guanidino-propoxy)guanidine
NOS	nitric oxide synthase

nNOS	neuronal nitric oxide synthase
iNOS	inducible nitric oxide synthase
OCT2	organic cation transporter 2
ODC	ornithine decarboxylase
PAO	polyamine oxidase
Pi	phosphate
PTP	permeability transition pore
PUT	putrescine
RBM	rat brain mitochondria
RCI	respiratory control index
RKM	rat kidney mitochondria
RLM	rat liver mitochondria
ROS	reactive oxygen species
SAM	S-adenosylmethionine
SAMDC	S-adenosylmethionine decarboxylase
SMO	spermine oxidase
SSAT	spermidine/spermine acetyltransferase
TPP^+	tetraphenylphosphonium
VDAC	voltage dependent anion channel
ΔE	electrode potential variation
$\Delta\Psi$	mitochondrial electric membrane potential
$\Delta\mu_{H}^{+}$	electrochemical gradient

Introduction

Biogenic amines are a class of compounds synthesized during normal metabolic processes in all organisms. Biogenic amines are biological regulators, including catecholamines, polyamines and agmatine [Toninello et al., 2004 a and b].

Polyamines

In eukaryotic cells, the polycationic polyamines, putrescine, spermidine and spermine are essential factors for embryonal development, differentiation and cell proliferation. The total intracellular concentration is very accurately regulated by metabolitc modulation or uptake in cells, and increases rapidly in proliferating or differentiating cells [Casero and Marton, 2007].

Polyamines are bound by weak interactions (electrostatic, dipole/dipole, cation/π, hydrogen bond) to various anion in the cell, including DNA, RNA, proteins and phospholipids. It follows that most of the interaction in which the polyamines are involved are reversible interactions. The main roles that polyamines show in the support of the cell growth and survival, are association with nucleic acids, maintenance of chromatin conformation, regulation of specific genes expression, ion-channels regulation, maintenance of membranes stability, and free-radical scavenging [Ha et al., 1998; Sava et al., 2006]. Moreover, polyamines activates kinases involved in mitochondrial signal transduction pathways [Toninello et al., 2004a].

Polyamine metabolism

Biosynthesis

The polyamine precursor is ornithine, a non-proteic amino acid, intermediate of urea cycle, and derived from arginine by the action of arginase (EC 3.5.3.1).

Ornithine decarboxylase (ODC, EC 4.1.1.17) is required for the first step in polyamine synthesis, in which ornithine is decarboxylated to produce putrescine. ODC is a pyridoxalphosphate-dependent enzyme and is the first rate-limiting step in polyamines biosynthesis. This enzyme is active as a homodimer with a very short half-life and is degraded by the 26S proteasome, without ubiquitination. The degradation of ODC is regulated by the antizyme, a small protein induced by polyamine that regulates also the polyamine transporter [Sakata et al., 2000].

Another rate-limiting step in polyamine biosynthesis is the decarboxylation of S-adenosylmethionine (SAM) by S-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50), that yields decarboxylated SAM which, in turn, donates its propyl amine moiety to form spermidine and spermine by two specific aminopropyl transferase, spermidine synthase (EC 2.5.1.16) and spermine synthase (EC 2.5.1.22). The SAMDC is a pyruvoyl-containing decarboxylase and its degradation is regulated by ubiquitination. On the contrary, spermidine and spermine synthase are constitutively expressed and are primarily regulated by the availability of their substrates.

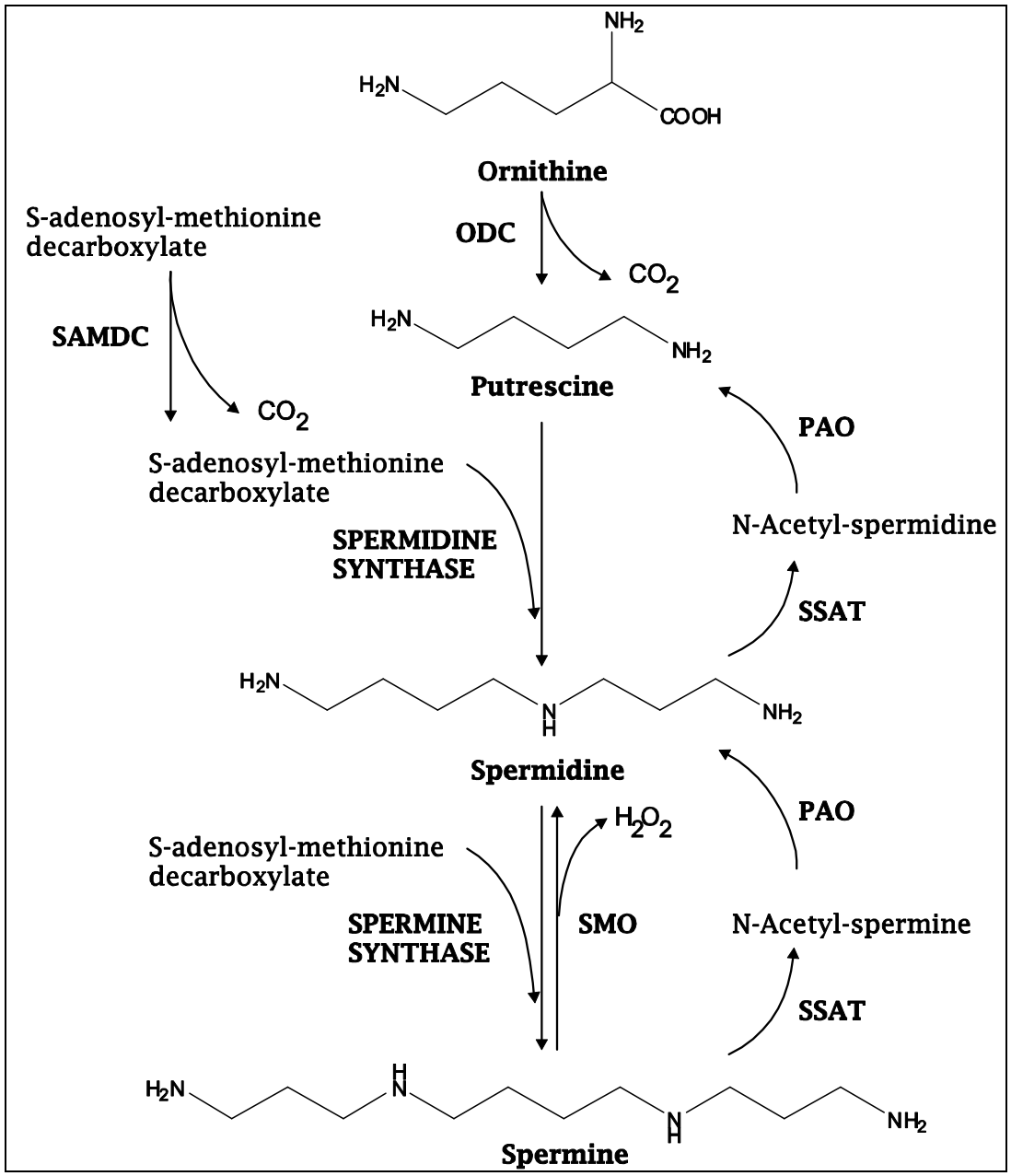


Fig. 1. Polyamine metabolism

Catabolism

Spermidine/spermine N^1-acetyltransferase (SSAT, EC 2.3.1.57) is a propylamine acetyltransferase that monoacetylates spermidine and may form either mono- or di-acetylates spermine. This inducible enzyme transfers an acetyl group from acetyl-coenzyme A to the N^1 position of spermidine or spermine.

These acetylated polyamines have two potential fates. First, diamines and acetylated polyamines could be exported by the putative diamine transporter, and eliminated in urine. Second, acetylated spermidine and spermine are also substrates for a flavin-dependent polyamine oxidase (PAO, EC 1.5.3.11), which catalyzes their conversion back to putrescine. The reaction produces spermidine or putrescine, depending on the starting substrate, 3-aceto-aminopropanal and H_2O_2. Recently, it has been characterized a new flavin-dependent enzyme, a spermine oxidase (SMO) that can oxidize non-acetylated spermine to produce spermidine, 3-aminopropanal and H_2O_2. [Vujcic et al., 2002].

Polyamine transport

Mammalian cells possess an energy-dependent and selective transport system involving an unidentified membrane transporter/carrier that is powered by a membrane potential and followed by a rapid accumulation into preexisting polyamine-sequestering vesicles [Hoshino et al., 2005]. This inducible and saturable transport system incorporates all three polyamines with appK_M values in the micromolar range. The transporter has high affinity for spermidine and spermine [Mitchell et al., 2007] and, as above-mentioned, is negatively regulated by antizyme.

Normally, the polyamines in excess are metabolized to acetyl derivatives, which are then excreted. Acetylpolyamines may be better substrates for excretion than polyamines themselves, although polyamines are better substrates for the uptake [Sakata et al., 2000]. Antizyme is able of inhibiting the uptake but also of stimulating polyamine excretion in order to maintain lower levels of polyamines.

Polyamines and mitochondria

Polyamines bind to mitochondrial membranes and are transported in the inner mitochondrial compartment by a specific transport system, sensitive to membrane potential [Toninello et al., 1992]. Some enzymes of polyamine metabolism are present in mitochondria (i.e. arginase, SSAT). In mitochondria, polyamines, in particular spermine, exhibit the different actions listed here:

- Increase in calcium accumulation, by rising the calcium affinity for its transporter, most probably to regulate free calcium concentration in the physiological range [Salvi et al., 2004].
- Inhibition of ATP hydrolysis catalyzed by F_0-F1-ATPase. In polyamine depleted cells the ATP content is lower than in normal cells, suggesting a possible role of spermine in maintaining the ATP concentration at high levels [Igarashi et al., 1989].
- Activation in the mitochondrial uptake of some enzyme, e.g. hexokinase, casein kinase CKII [Toninello et al., 2004a].
- Stimulation of pyruvate dehydrogenase complex activity [Pezzato et al., 2008].
- Free radical scavenging action in isolated mitochondria and inhibition of mitochondrial permeability transition [Sava et al., 2006].
- Induction of apoptosis through mitochondrial-mediated pathway in different cell types in conditions of polyamine depletion [Nitta et al., 2002; Gardini et al., 2001]. This effect may be prevented by over-expression of the anti-apoptotic protein Bcl-2 [Holst et al., 2008].

Agmatine

The term "agmatine" was coined in 1910 by Albrecht Kossel, who first identified the substance in herring sperm [Kossel, 1910]. It was discovered in many plants, bacteria and invertebrates in following years. Later, agmatine was shown to occur also in mammals [Raasch et al., 1995], by identifying its presence in bovine brain also by recognizing it with the clonidine-displacing substance, a compound previously studied by other groups [Atlas et al., 1984; Li et al., 1994]. In fact, agmatine is able to displace clonidine from α_2-adrenergic and imidazoline receptors and mediate an anti-hypertensive effect. After its discovery in brain, agmatine was revealed also in stomach, aorta, liver, lung, heart, kidney, spleen and plasma where its concentration is similar to that of catecholamines (0.45 ng/ml) [Raasch et al., 1995].

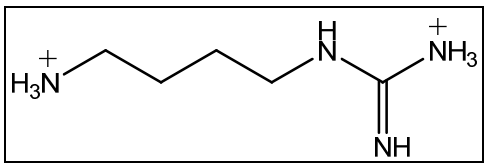


Fig. 2. Agmatine

Agmatine [1-(4-aminobutyl)guanidine] is a biogenic amine mainly present in the diprotonated form at physiological pH and produced by decarboxylation of arginine by the enzyme arginine decarboxylase (ADC). Agmatine has different physiological roles, notably it behaves as a neurotransmitter [Regunathan and Reis, 2000], and regulator of polyamine concentration [Isome et al., 2007].

Agmatine metabolism

Biosynthesis

In bacteria and plants agmatine is produced by ADC (EC 4.1.1.19). The presence of this enzyme in mammals is still debated.

In *E. coli* ADC exist in constitutive (biosynthetic) and inducible (biodegradative) isoforms that are both cytosolic and their activities are dependent on pyridoxalphosphate and Mg^{2+}. The putative mammalian ADC is probably associated with mitochondrial membranes and is able to decarboxylate both arginine and ornithine. ADC activity is inhibited by Ca^{2+}, Co^{2+}, and polyamines. The ADC activity is constitutive in mammalian cells and high in confluent cells, so it may serve as a constitutive source of polyamines [Regunathan and Reis, 2000].

Catabolism

Agmatine is degraded by two distinct ways depending on the tissue where it is contained:

- By diamine oxidase (DAO, EC 1.4.3.6) in peripheral tissues, which catalyzes the degradation to guanidinobutyraldehide, then dehydrogenated and hydrolyzed by specific enzymes and finally excreted from the body. The heterogeneous location of DAO suggests that certain tissues or organs may have the capacity to regulate local agmatine levels [Lortie et al., 1996].
- By agmatinase activity (EC 3.5.3.11, agmatine ureohydrolase) which catalyzes the formation of urea and putrescine, in brain. Agmatinase is the only enzyme specific for agmatine catabolism. The enzyme require Mn^{2+} as cofactor in the active site and possesses a mitochondrial target sequence in the N-terminal of the protein.

Agmatine transport

Absorption of agmatine produced by bacteria flora of the gastro-intestinal tract or introduced by the diet is another factor that influences agmatine homeostasis in the mammalian organism. In rat, about 60% of agmatine taken up from the stomach and intestine is accumulated in the liver. This organ plays a crucial physiological role in the maintenance of agmatine homeostasis in organism [Haenisch et al., 2008]. Agmatine is transported in tissues by an energy-dependent mechanism which is reduced by simultaneous, dose-dependent, administration of putrescine, suggesting a correspondence between the transport mechanism of polyamines and agmatine, probably using a carrier [Molderings et al., 2002]. The amine is absorbed from the lumen of the gastrointestinal tract and distributed into the various tissues via the circulating blood.

Agmatine does not cross cellular membrane by simple diffusion, but requires the presence of a channel or transporter protein in plasma membrane, because of its positive charges [Grundemann et al., 2003]. Agmatine is transported in cells as a molecule with a single positive charge, and the probable transporter has been identified as the extraneuronal monoamine transporter (EMT) or organic cation transporter 2 (OCT2) [Grundemann et al., 2003].

Agmatine action in polyamine metabolism

Agmatine regulates polyamine metabolism by acting on different enzymes involved in the polyamine pathway. It competitively inhibits nitric oxide synthase (NOS), that catalyze the reaction:

L-arginine + NADPH + H^+ + O_2 ⇄ citrulline + NO + $NADP^+$

thus evidencing an important role in modulating NO production as an endogenous regulator [Galea et al., 1996]. Particularly, agmatine irreversibly inhibits the neuronal NOS (nNOS) and downregulates the inducible form (iNOS), exhibiting a neuroprotective role [Halaris and Plietz, 2007].

Agmatine induces SSAT and antizyme of ODC, which, as above mentioned, inhibits both enzyme activity and polyamine transporter. Finally, it is metabolized by agmatinase to form urea and putrescine, hence it is considered a polyamine precursor.

Agmatine as neurotransmitter/neuromodulator

Agmatine, due to its interaction at neuronal level, may be considered as a neurotransmitter or neuromodulator because exhibits various effects typical of these compounds [Halaris and Plietz, 2007].

- It is synthesized in neurons: the putative ADC is localized in neurons and glia, and agmatine concentration in brain is similar to that of other neurotransmitters (0.5 μg/g of wet tissue).
- It is released presinaptically by a Ca^{2+}-dependent depolarization and is co-packaged in synaptosomes with glutamate and vasopressin. The distribution of agmatine in brain is differentiated.
- It binds to some receptors: α_2-adrenergic, imidazoline, N-methyl-D-aspartate (NMDA), serotonin 5-HT_3, nicotinic and voltage-gated.
- A specific uptake of agmatine is present in plasma membrane. The transporter is the same of polyamines, on which agmatine exhibits a dose-dependent inhibition [Satriano et al., 2001]. Moreover, agmatine is taken up into synaptosomes.
- It is inactivated by a specific mechanism involving agmatinase.

Agmatine also induces the release of catecholamines from adrenal chromaffin cells, promotes gastric acid secretion, and stimulates the release of insulin from β pancreatic cells. It may also regulate emotion and the function of opiate receptor, and enhance the analgesic effect of morphine [Qiu and Zheng, 2006; Regunathan, 2006]. Indeed, agmatine exhibits a neuroprotective role mainly by inhibiting nNOS, since NO contributes to ischemic brain injury.

Agmatine action in different organs

Liver

Agmatine, in rat hepatocytes, is taken up by a high-affinity system in a nonsaturable process which transports also putrescine. Moreover, hepatocytes are also capable of synthesizing agmatine. Thus the liver appears to play a crucial role in the regulation of agmatine in the systemic circulation [Haenisch et al., 2008]. In hepatocytes, agmatine decreases polyamine content by inducing SSAT and only a small amount (10%) is transformed in putrescine by agmatinase activity. The main pathway in rat liver cells for agmatine catabolism is through DAO activity [Cabella et al., 2001]. The still unreacted molecule most probably acts by inducing apoptosis with the involvement of mitochondria [Gardini et al., 2001].

Furthermore, in a system of liver perfusion, agmatine was able to stimulate β-oxidation and up-regulate ureagenesis [Nissim et al., 2006].

Kidney

Agmatine concentration in kidney is very high, for example in rat kidney reaches a values over 400 μM [Isome et al., 2007].

The DAO in renal tissues appears to be primarily located in glomerular structures. Imidazoline receptors have been localized to the basolateral aspect of proximal tubules. In kidney membrane preparations, agmatine stimulates Na^+/K^+ ATPase [Lortie et al., 1996]. Again in kidney, agmatine behaves as a functional regulator: increases the rate of proximal tubule filtration and glomerular reabsorption.

In hyperglycemic mesangial cells agmatine are able to protect against hydrogen peroxide production. Since reactive oxygen species (ROS) injure cells when in excess of normal levels, contributing to the pathogenesis of many renal diseases, agmatine could be considered as a protective agents [Lee et al., 2003]. Moreover, in a model of glomerulonephritis, agmatine is able to suppress proliferation of mesangial cells and so it is proposed as a drug candidate for the treatment of human mesangial proliferative glomerulonephritis [Eto et al., 2006].

Brain

Agmatine exhibits antidepressant, anxiolytic, antinociceptive, anticonvulsive, and neuroprotective effects in brain [Halaris and Plietz, 2007]. Moreover, age-related changes in agmatine levels in memory-associated brain structures, and a potential involvement of

agmatine in the aging process have been demonstrated [Liu et al., 2008].

As above mentioned, agmatine is a potential neuroprotective agent and exerts neuroprotection against ischemia-hypoxia injury, and glutamate-induced neurotoxicity, by activating imidazoline receptors, blocking NMDA receptor, inhibiting all isoforms of NOS, and selectively blocking the voltage-gated calcium channels [Qiu and Zheng, 2006].

Agmatine action on cultured cells

Agmatine presence in cultured media usually induces polyamine depletion in cells by causing proliferation suppression or apoptosis, depending on proliferative status of cells. Agmatine exhibits a cytostatic effect in proliferative cells without apoptosis induction [Gardini et al., 2003], whereas apoptosis is induced in non-proliferative cells [Gardini et al., 2001].

In rat hepatocyte cultures, indeed, agmatine promotes apoptosis through release of cytochrome c from mitochondria and activation of caspase-3 [Gardini et al., 2001]. Inhibition of polyamine biosynthesis and transport by agmatine results in suppression of proliferation in a trasformed cell line from mouse kidney proximal tubule (MCT) [Satriano et al., 1998], as happens also in rat hepatoma cell lines (HTC) [Gardini et al., 2003]. The inhibition of proliferation also in intestinal tumor permits to hypothesize for agmatine an antineoplastic role and it is likely that this activity can be ascribed to the regulation of polyamine homeostasis by agmatine [Molderings et al., 2004]. Agmatine intracellular inhibition of cell proliferation is associated with a reduction of polyamine levels by ODC inhibition. The ODC can be regulated by agmatine at translational level, in addition to its posttranslational regulation of antizyme [Wolf et al., 2007].

Agmatine and mitochondria

Agmatine is found in mitochondria together with its metabolic enzymes, ADC and agmatinase [Regunathan and Reis, 2000]. The imidazoline receptor I_2 is also located on a domain of monoamine oxidase A (MAO) in rat liver mitochondria [Anderson et al., 2006]. Moreover, agmatine stimulates oxygen consumption, β-oxidation and ureagenesis in a liver perfusion system and in isolated mitochondria [Nissim et al., 2006]. As above mentioned, in rat hepatocyte cultures agmatine induces mitochondrial swelling, release of cytochrome c and ensuing apoptosis.

Materials and Methods

Mitochondria isolation

Mitochondrial preparation was performed using the modified method of Schneider (1950), by conventional differential centrifugation. Rat liver mitochondria (RLM), rat kidney mitochondria (RKM) and rat brain mitochondria (RBM) were prepared with the following method.

The organs, liver, kidney and brain, were taken from Wistar rats of 180gr weight (after 16 hours fasting for liver mitochondria) minced and washed in an isolation medium:

- For RLM and RKM: 250 mM sucrose, 5 mM Hepes and 2 mM EGTA (pH 7.4).
- For RBM: 320 mM sucrose, 5 mM Hepes and 0.5 mM EDTA (pH 7.4).

After washing out the blood, the minced organ was treated in Potter homogenizer. The homogenate was centrifuged in a Beckman J2-21 centrifuge, with Ja-17 rotor, cooled at 0-5°C. The first low-speed centrifugation, at 2300 rpm (755 g) for 5 min, is used to remove nuclei and intact cells. The supernatant, that contains mitochondria, microsomes and cytosol, was subjected at a second centrifuge at 9000 rpm (10800 g) for RLM and RKM, at 11000 rpm (15900 g) for RBM, for 10 min.

After this step, the mitochondrial preparations were different for the various organs:

- Liver and kidney mitochondria precipitates and were washed in a final centrifuge at 11000 rpm (15900 g) for 5 min, in medium without EGTA. Finally, they were resuspended in 2 ml of final medium and are ready to use.
- Brain mitochondria, instead, were ultracentrifuged in a Ficoll gradient (12-9-6%) at 23500 rpm (75000 g) for 45 min, in a Beckman Optima L-90K Ultracentrifuge, with SW40Ti rotor, cooled at 0-5°C, to eliminate all contaminant synaptosomes. Then, the precipitate containing mitochondria was centrifuged to wash out EDTA in a medium devoid of it at 11000 rpm for 5 min.

Protein content determination

The protein content of the mitochondrial suspension, was measured by the biuret method with bovine serum albumin as standard [Gornall et al., 1949]. The method uses the formation of a violet complex between rameic ion and amidic nitrogen of protein (biuret reaction).

The Gornall's solution contains:

- Rameic sulphate ($CuSO_4 \cdot 5\ H_2O$) 1.5 g/l
- Sodium and potassium tartrate ($NaKC_4H_4O_6 \cdot 4\ H_2O$) 6 g/l
- Sodium hydroxide (NaOH) 30 g/l

The solution is photosensible and is conserved at dark.

After the reaction, the samples was readed at 540 nm in an UV/VIS KONTRON UVIKON 922 spectrophotometer, and the measure was performed twice respect a blank without mitochondria.

Standard medium for mitochondrial measurement

Mitochondria (1 mg/ml) was incubated in the following medium:

- 200 mM sucrose
- 10 mM Hepes
- 5 mM succinate
- 1.25 μM rotenone
- 1 mM phosphate.

The medium was at pH 7.4 and 20°C. All variations or additions at this medium will be reported in the appropriate captions in the result section.

Transmembrane potential measurement with ionoselective electrode

The transmembrane electric potential ($\Delta\Psi$) was measured using a specific electrode on the basis of distribution of the lipid-soluble cation tetraphenylphosphonium (TPP^+) [Affolter and Sigel, 1979; Kamo et al. 1979].

The electrode is a complex semipile formed by an anion reversible electrode, at Cl^- (inner reference electrode), inserted in a case containing a TPP^+Cl^- solution at known concentration, at contact with a TPP^+ permeoselective membrane. This semipile exhibits an electric potential that changes with logarithm of TPP^+ activity in the sample. It is coupled with an electrode at constant potential (outer reference

electrode) and formed a pile, with an electroengine force/power that results linear function of activity logarithm of ion to measure.

The membrane separates two solution at different concentration of the same electrolyte (TPP^+Cl^-): TPP^+ is the counterion and Cl^- is the coordinate ion of the membrane, because their charges are respectively opposite and equal to the charge in the membrane structure, gived by tetraphenylborate (TPB^-). The membrane is a cationic membrane, because is permeable to TPP^+.

The pile exhibits a potential variation (ΔE), that is measure of the proceed of the process of counterions transfer from high concentration solution c'' (fixed) to lower concentration solution c' (unknown), with c''>c'.

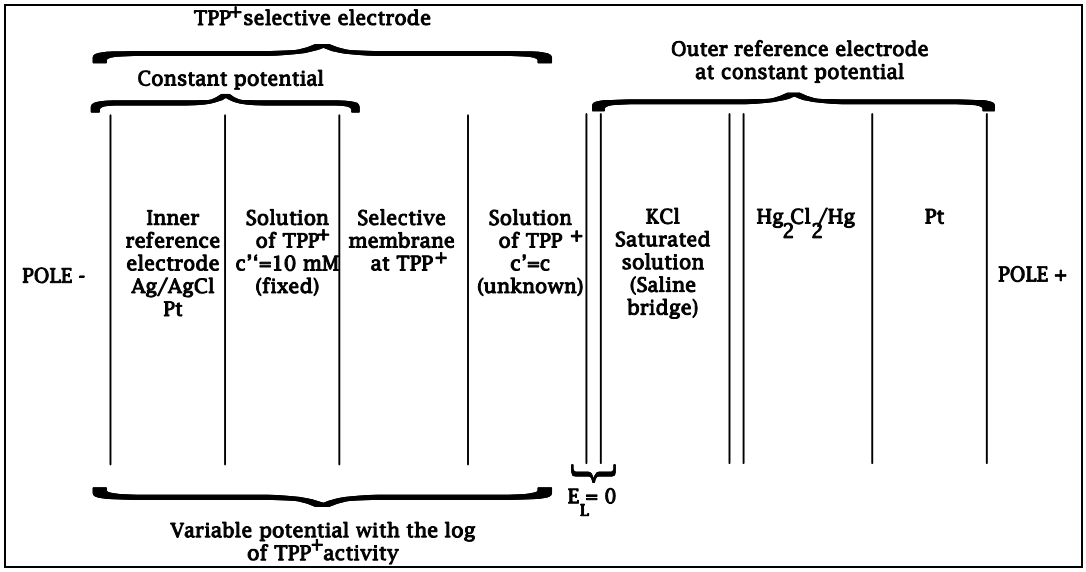


Fig. 3. TPP^+ selective electrode.

The inner and outer electrode potentials is constant, and the potential difference of the pile is determined only by membrane potential (function of the ratio between unknown and known concentration of the counterion TPP^+) and interliquide potential (E_L), minimized by KCl saturated saline bridge.

The ΔE results:

$$\Delta E = E_1 - E_2$$

$$E_1 = E_{k'} + \Delta E_X$$

$$E_2 = E_{k''} + E_L$$

Where:

E_1 = ionoselective electrode potential

E_2 = outer reference electrode potential + interliquide junction potential

$E_{k'}$ = inner reference electrode potential

ΔE_x = ionoselective membrane potential difference

$E_{k''}$ = outer reference electrode potential

E_L = interliquide junction potential ≈0

$$\Delta E = E_{k'} + \Delta E_X - E_{k''} - E_L$$

$$\Delta E = E_{k'} - E_{k''} + \Delta E_X$$

Setting: $E_{k'} - E_{k''} = z$ (constant)

Then:

$$\Delta E_X = E_0 + \frac{2.3RT}{nF}\log c'' - E_0 - \frac{2.3RT}{nF}\log c'$$

Setting: $E_0 + \frac{2.3RT}{nF}\log c'' = U$ (constant)

The ionoselective membrane potential difference (ΔE_x) becomes:

$$\Delta E_X = U - \frac{2.3RT}{nF}\log c'$$

And pile ΔE results:

$$\Delta E = z + U - E_0 - \frac{2.3RT}{nF}\log c'$$

Finally, setting: $z + U - E_0 = K$, it is obtained:

$$\Delta E = K - \frac{2.3RT}{nF} \log c' \qquad [1]$$

Where:

K = constant resulting from the algebraic sum of all the constant potentials in the electrode

F = 96485 coulombs mol^{-1} = 23.06 Kcal $volt^{-1}$ mol^{-1}

R = 8.341 Joule mol^{-1} K^{-1}

T = 20°C

The electrode response is linear with the logarithm of TPP^+ concentration, with an increase of about 58 mV every ten unit of variation in the TPP^+ concentration, until the concentration decrease at 10^{-7} M, according to Nernst's equation.

The $\Delta\Psi$ is determined measuring the TPP^+ distribution across the mitochondrial membrane with the electrode. The mitochondrial membrane is permeable to TPP^+, that distributes according to Nernst's equation:

$$\Delta\psi = \frac{2.3RT}{nF} \log \frac{[TPP^+]_{out}}{[TPP^+]_{in}} = 58 \log \frac{[TPP^+]_{out}}{[TPP^+]_{in}} \qquad [2]$$

Where:

$[TPP^+]_{out}$ = outer TPP^+ concentration

$[TPP^+]_{in}$ = inner TPP^+ concentration

Determining the variation of electrode ΔE.

Considering the law of mass conservation:

$$V[TPP^+]_{out} + v[TPP^+]_{in} = V[TPP^+]_0 \qquad [3]$$

Where:

V = medium volume containing 1 mg of mitochondrial proteins (1 ml in our system)

v = volume of inner mitochondrial space corresponding to 1 mg of mitochondrial proteins (≈ 1 µl)

$[TPP^+]_0$ = TPP^+ concentration before mitochondrial addition

Equation [3] is inserted in [2] and ΔE is correlated to $[TPP^+]_{out}$, with some mathematic passages, it is obtained:

$$\Delta\psi = 58\log\frac{v}{V} - 58\log\left(10^{\left(\frac{\Delta E-\Delta E_0}{58}\right)} - 1\right) \qquad [4]$$

Where:

ΔE_0 = electrode potential difference before mitochondrial addition

In order to calculate correctly the ΔΨ it is necessary to know the v/V ratio, that is the v value. If mitochondrial volume does not vary during experiment, the 58 log v/V remains constant.

The v value was calculated using the [^{14}C]sucrose distribution [Palmieri e Klingenberg, 1979], and it corresponds to 1 μl/mg of mitochondrial proteins. Jensen et al. (1986), comparing the ΔΨ value obtained measuring the ^{86}Rb and that measuring with electrode, propose to correct with the subsequent equation:

$$\Delta\psi_{Rb} = \frac{(\Delta\psi_{el} - 66.16mV)}{0.92} \qquad [5]$$

In which $\Delta\Psi_{el}$ is the value obtain in [4].

Before to proceed in ΔΨ measure, it is performed the electrode calibration, to determine experimentally the ratio $\frac{2.3RT}{nF}$

The ratio corresponds to the slope of the line:

$$\Delta E = K - \frac{2.3RT}{nF}\log c'$$

The calibration was performed in the incubation condition, without mitochondria, adding TPP^+ and measuring the electrode variation. The final concentration of TPP^+ should not exceed 1-2 μM, because an excessive amount of TPP^+ can later depolarize the mitochondrial membrane.

The slope calculation was derived graphically: the measured ΔE values are carried in function of log[TPP^+]. The ΔE corresponding to an increase in TPP^+ concentration of ten times is extrapolate. The theoretical value, according to Nernst, corresponds to 58 mV.

After calibration, mitochondria were added to incubation medium, TPP^+ enters across mitochondrial membrane and distributes between

medium and matrix, according to Nernst's equation. A potential difference at the electrode was originated respect to value reached after calibration, named ΔE-ΔE_0, and registered by the recorder as a deflection (decrease of TPP^+) concentration in the medium. The fitting of this potential difference variation in the [4] allow to obtain the $\Delta\Psi$ value.

Uptake of agmatine in mitochondria

Mitochondria were incubated in the standard medium in presence of radiolabeled agmatine (50 μCi/mmol). At the end of incubation time, the samples were collected and centrifuged on a 12% sucrose/silicon gradient. The silicone was removed and the samples were washed and solubilized. Finally, the radioactivity incorporated in mitochondria was counted with a specific scintillator (Liquid Scintillation Analyzer Packard 1500) with scintillation liquid (Packard).

Oxygen consumption measurement by Clark's electrode

The electrode is composed by a platinum cathode and silver/silver chloride reference anode. These electrodes are immersed in a saturated KCl solution and separated from the reaction vessel by a Teflon membrane that is permeable to oxygen. When a potential difference of 0.6-0.8 mV is applied, electrons are generated at the anode and utilized at cathode for oxygen reduction.

Reaction at the electrodes:

$$\text{anode: } 4Ag + 4Cl^- \longrightarrow 4AgCl + 4e^-$$

$$\text{cathode: } O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$$

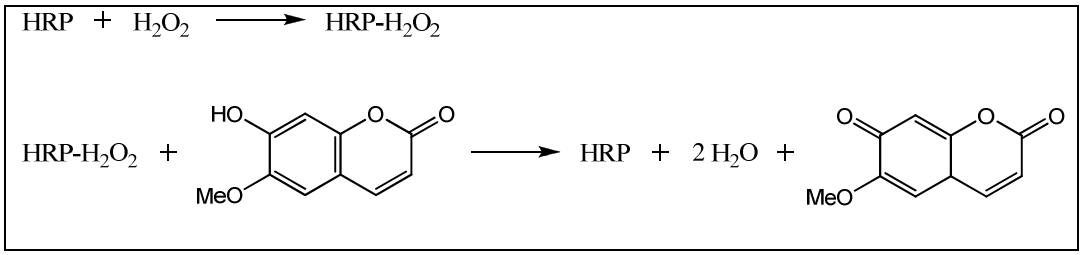
The overall result is that of a transfer of electrons from the cathode to the anode occurs, causing a current to flow between the two electrodes which can be measured in a external circuit and and drawn in a curve that measure the current intensity in the time. The current is proportional to the partial pressure of oxygen in the sample.

The measurement of the respiratory control index (RCI) and of the ADP/O ratio, are done by addition of 200 μM ADP to the mitochondrial suspension. The ratio of oxygen uptake in state 3 (in presence of ADP) is divided by the rate of oxygen uptake in state 4 (in absence of ADP) to obtain the ICR. Instead, the ADP/O ratio is the ratio of the nmol of added ADP divided by the nanoatoms of oxygen utilized during state 3 respiration.

Fluorimetric assay to hydrogen peroxide determination

The hydrogen peroxide produced by mitochondria is measured by scopoletin (6-metoxy-7-idroxy-1,2-benzopirone) method [Loschen et al., 1971]. The mitochondria were incubated in presence of 1 μM scopoletin and 10 μM horseradish peroxidase (HRP).

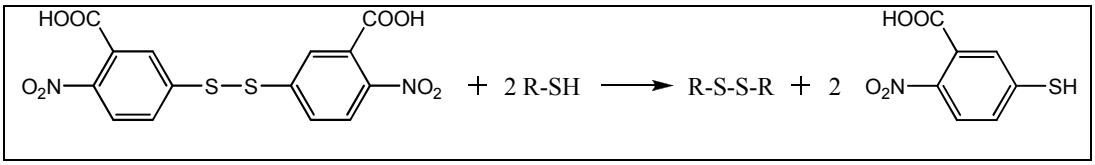
The HRP, in presence of hydrogen peroxide, oxidizes scopoletin that loses its fluorescence, with the reaction:



The fluorescence was measured utilizing a SHIMADZU RF-5000 spectrofluorimeter, with 350 nm of excitation wavelength and 460 nm of emission wavelength.

Redox state determination of sulfhydryl groups

The determination of sulfhydryl groups is performed by Elmann method (1959), modified by Bindoli and Rigobello (2002), utilizing the 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) as –SH groups indicator. The DTNB reacts with reduced sulfhydryl groups, separating in two molecules of carboxy-nitro-thiophenole (CNTP), as in reaction:



After the specific incubation, the samples were centrifuged at 12000rpm in a Centrifuge 5415C, and washed to eliminate the incubation medium. The mitochondrial fraction present in medium was resuspendend in a solubilization medium (EDTA 10 mM, Tris 0.2 M, SDS 1%, pH 8.3).

The sample absorbance was read after 1 mM DTNB addition in a UV/VIS KONTRON UVIKON 922 spectrophotometer, at 412 nm of wavelength. The sulfhydryl groups concentration was obtained by Lambert-Beer law, with ε=13600 $M^{-1}cm^{-1}$ for DTNB.

The final concentration of sulfhydryl groups was expressed as percentage of the starting amount using the control as reference (100% reduced sulfhydryl groups).

Redox state determination of pyridine nucleotides

The redox state of pyridine nucleotides was measured as the fluorescence variation of NAD(P)H/$NAD(P)^+$ ratio, utilizing a SHIMADZU RF-5000 spectrofluorimeter, with 354 nm of excitation wavelength and 462 nm of emission wavelength.

Mitochondrial swelling determination

Mitochondrial swelling occurs when solute enters in high quantity in mitochondrial matrix, e.g. when mitochondrial permeability transition happens, causing an increase in the osmotic pressure.

This phenomenon can be measure by "light scattering" technique that consists in the ray of light dispersion when it crosses the mitochondrial suspension. If matrix volume is increased, it can be observed a decrease of dispersion and, consequently, a decrease also of absorbance.

Swelling was monitored using an UV/VIS KONTRON UVIKON 922 spectrophotometer, at 540 nm of wavelength.

[^{14}C-guanide]agmatine synthesis and purification

The agmatine used in experiment for transport in mitochondria or as standard for amidinotransferase activity, was synthesized from [^{14}C-guanide]arginine (Amersham). The reaction buffer contains 0.2 M sodium acetate, 5 mM pyridoxalphosphate, 0.1% bovine serum albumin (BSA), pH 5.2. At this buffer were added 18 mM [^{14}C-guanide]arginine (0.6 mCi/mmol) and 1 U of *Escherichia coli* ADC, and incubated at 37°C for 5 h. The reaction was stopped adding 5 M KOH. The purity of preparation was assayed by HPLC method.

[^{14}C-guanide]agmatine synthesis in proximal tubule cell line (MCT)

Cells were cultured in complete DMEM High glucose medium, supplemented with 10% FBS and 100 U penicillin/streptomycin until 70% confluence, then they were starving O.N. in presence of 25 μM methylglyoxal bis(guanylhydrazone) (MGBG) or 5 mM difluoromethylornithine (DFMO). The medium were substituted after 12 h with a complete medium containing also 0.1 μCi of [^{14}C-guanide]arginine (0.60 mCi/mmol). After 24 h of incubation, cells were washed twice with PBS and collected with TCA 5%. The suspension was centrifuged at 500 g for 5 min in a microcentrifuge, and the supernatant was basified with 3 M KOH and was treated with butyl alcohol to extract agmatine. The butanolic phase was extracted and was evaporated to dryness *in vacuum*. The samples were finally resuspended in acetonitrile and were prepared to HPLC analysis.

Amidinotransferase purification

The purification of RKM lysate (prepared using isolated RKM treated with 1% Triton X100) was performed with a FPLC method using a DEAE-52-Cellulose column (Pharmacia). All steps of purification were carried at 4°C. Column was washed in a buffer containing 27.5 mM TRIS pH 7.4, 0.1 mM EDTA, 2 mM β-mercaptoethanol at flux of 1 ml/min. The protein content was monitored measuring absorbance at 280 nm. When absorbance was <0.05 units, the sample were eluted in a linear gradient of NaCl to reach concentration of 0.2 M in 45 min. After column fractions of 5 ml were collected and the fractions with more protein were desalted with ultrafiltration. These fraction were used for the arginine:putrescine amidinotransferase activity assay.

The protein content after purification was evaluated also in SDS-PAGE with a 10% acrylamide gel and Comassie blue coloration.

Arginine:putrescine amidinotransferase activity assay

RKM lysate or purified fraction was suspended in a medium containing 0.1 M TRIS pH 9.0, and 0.1 mM EDTA. The incubation was performed at 37°C in different times, described in the respective figures. In the incubation buffer were also present 30 μM amidinoguanidine to inhibit DAO, and 2 mM ditiothreitol (DTT).

In order to determine the total and specific activity, the samples were incubated with different cold putrescine concentrations in presence of 100 mM [^{14}C-guanidine]arginine (1.20 μCi). At the end of the incubation time, the reaction was stopped with TCA 5% and samples were centrifuged at 20000 g for 10 min in a Biofuge 28RS (Heraeus). Supernatant was finally prepared to HPLC analysis.

Qualitative determination of agmatine with chromatographic HPLC method

The radiolabeled molecules were detected using a HPLC chromatographic method using a inverse phase column (Spherisorb 5 μM C-18, Waters). The column was equilibrated in a buffer containing 140 mM sodium acetate, 17 mM triethilamine (TEA) and 10 mM octane sulfonate, pH 4.5 with fosforic acid, at flow 1ml/min. At this acid pH, agmatine is completely protonated and positively charged, the octane sulfonate acts as counterion for agmatine, forming an apolar complex. The elution was performed by using a linear gradient up to the 100% of the elution buffer (70% acetonitrile in water) in 20 min, the flow was increased until 1.5 ml/min and was remained constant for 30 min. The detection of radiolabeled peak was carried out using a specific scintillator (Packard) for HPLC with FLO SCIN II scintillation liquid (Packard).

1. Uptake of agmatine in mitochondria

Polyamine transport in mitochondria

As above mentioned, polyamines are transported in cells by a specific energy-dependent mechanism. Moreover, polyamines are transported also in mitochondria by a specific uniporter [Toninello et al., 1988 and 1992].

Polyamines, in particular spermine, are transported bidirectionally across the mitochondrial inner membrane in liver mitochondria, after the binding at two distinct binding sites in membrane. The influx, which occurs electrophoretically, is dependent on a high transmembrane potential and exhibits a non-linear current/voltage relationship. The uniporter is a channel common for all the three natural polyamines. The transport is a saturable system in which its affinity increases with the charge of the substrate. Moreover, polyamine uptake in mitochondria is not shared with amino acids [Toninello et al., 1992].

Agmatine transport in mitochondria

Agmatine has been individualized in mitochondria as well as its metabolic enzymes and the imidazoline receptor which binds the amine. These observations permit to hypothesize the existence of a specific transport system for agmatine in inner mitochondrial membrane that could explain a possible physiological role for agmatine within mitochondria. Furthermore the existence of the polyamine transport in mitochondria suggests the possibility of a common transporter with agmatine in these organelles, as demonstrated in cell membranes in which the transport is shared with polyamines and depends on membrane potential.

This first part of this thesis investigates about the transport of agmatine in mitochondria in order to characterize its mechanism and the similarity with the polyamine transporter. Moreover, it is reported also a comparison between agmatine transport in liver, kidney and brain mitochondria.

Results

Agmatine structure-activity relationship

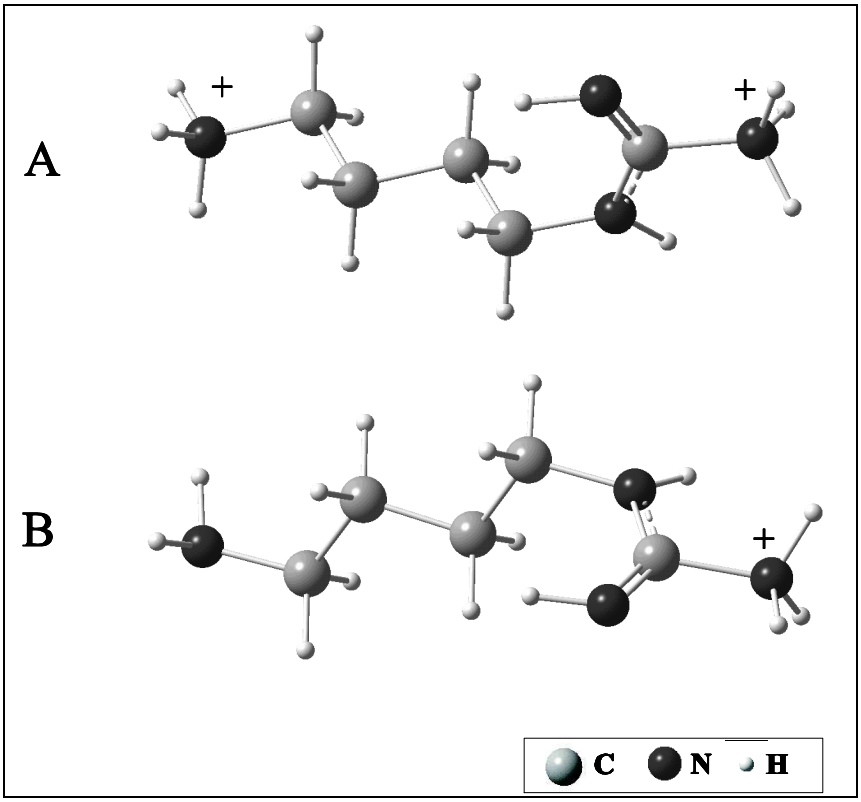


Fig. 4. Agmatine structure.
A: divalent cation; B: monovalent cation. Structures were determined by *ab initio* calculations coupled to Raman spectroscopy [Toninello et al., 2006].

At physiological pH, agmatine is a diamine with two net positive charges (fig. 4), since it has the lower pKa value of 9.07 [Grundemann et al., 2003]. Thus, it may be considered as a divalent cation. At high pH, probably present in the microenvironment of the transport system, a monovalent form can be also present.

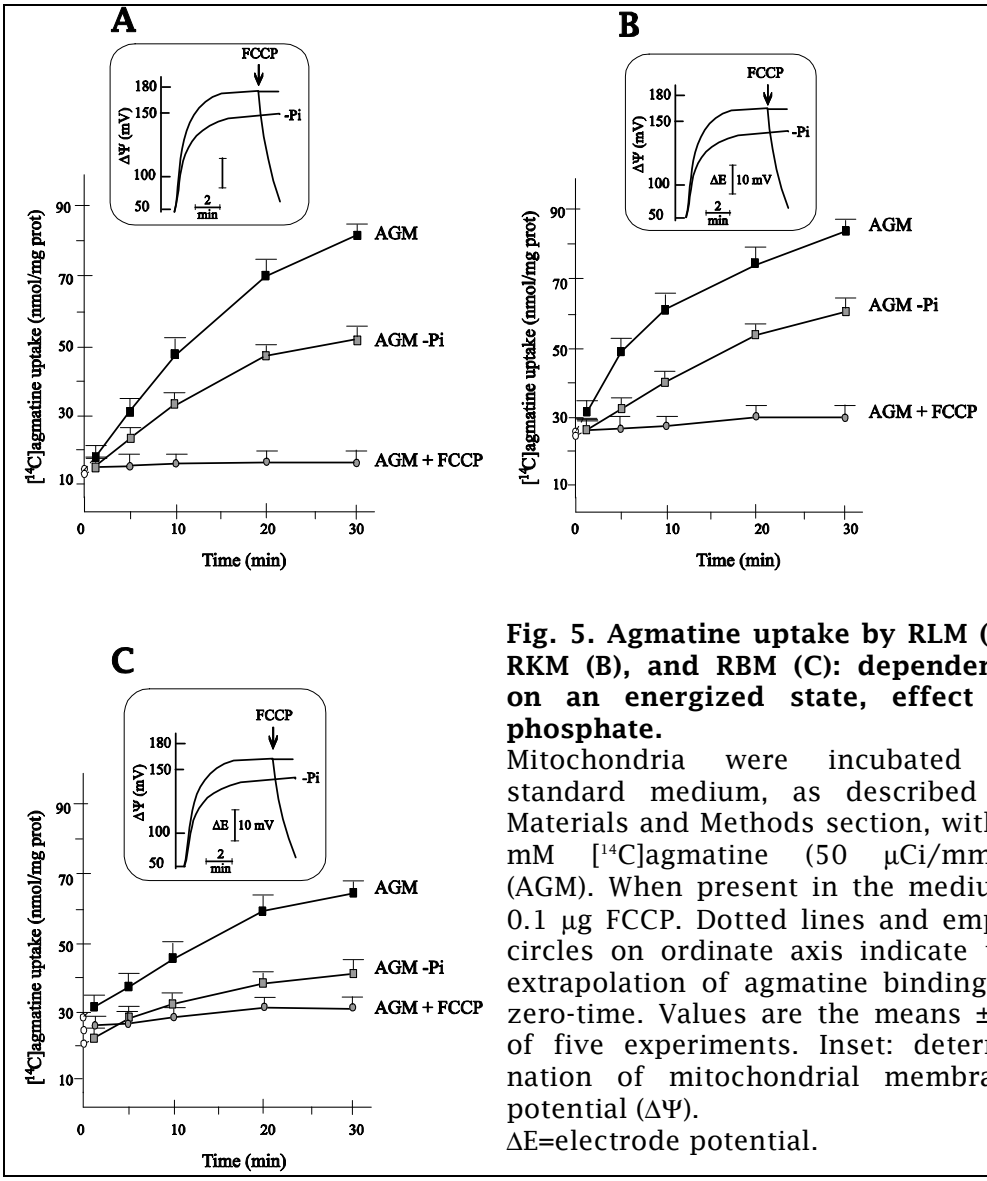


Fig. 5. Agmatine uptake by RLM (A), RKM (B), and RBM (C): dependence on an energized state, effect of phosphate.
Mitochondria were incubated in standard medium, as described in Materials and Methods section, with 1 mM [^{14}C]agmatine (50 μCi/mmol) (AGM). When present in the medium: 0.1 μg FCCP. Dotted lines and empty circles on ordinate axis indicate the extrapolation of agmatine binding at zero-time. Values are the means ±SD of five experiments. Inset: determination of mitochondrial membrane potential (ΔΨ).
ΔE=electrode potential.

Agmatine uptake (measured with radiolabeled molecule) by energized RLM incubated in standard medium, is of about 80 nmol [^{14}C]agmatine/mg protein in 30 min of incubation (fig. 5 A), RKM took up 85 nmol/mg protein (panel B) and RBM 60 nmol/mg protein (panel C). In the presence of FCCP (carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone), an uncoupler of mitochondrial respiration which completely collapses the electric membrane potential (ΔΨ) (see insets in Fig. 5), agmatine uptake is completely inhibited in mitochondria from all the three organs. In the absence of phosphate (Pi), ΔΨ exhibits a lower value (fig. 5, insets). This because phosphate, when present, by collapsing ΔpH gradient, shifts ΔΨ to a higher value. In this condition (without Pi) also the uptake of the

amine is lower than in presence of the anion. It reaches only about 50 nmol/mg protein in RLM, 60 nmol/mg protein in RKM, and 40 nmol/mg protein in RBM (fig. 5). Thus, agmatine transport depends on the energizing state of mitochondrial membrane.

Characterization of agmatine transport in RLM and RKM

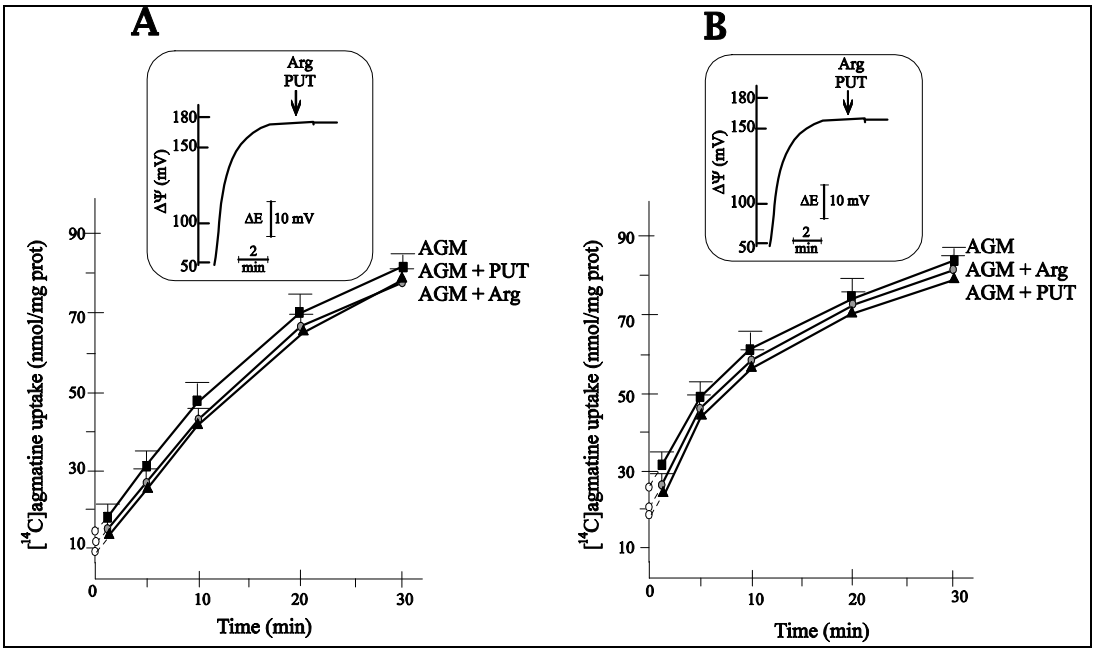


Fig. 6. Effect of polyamine and amino acids on agmatine uptake in RLM (A) and RKM (B).
Mitochondria were incubated in standard medium, as described in Materials and Methods section, with 1 mM [^{14}C]agmatine (50 μCi/mmol) (AGM). When present in the medium: 1 mM putrescine (PUT), 1 mM arginine (Arg). Empty circles on the ordinate axis indicate agmatine bound at zero-time. Values are the means ±SD of five experiments. Inset: determination of ΔΨ. ΔE=electrode potential.

The addition of putrescine or arginine in the incubation does not inhibit agmatine uptake (fig. 6), so agmatine transporter is not the same of polyamines or amino acids in liver and kidney mitochondria.

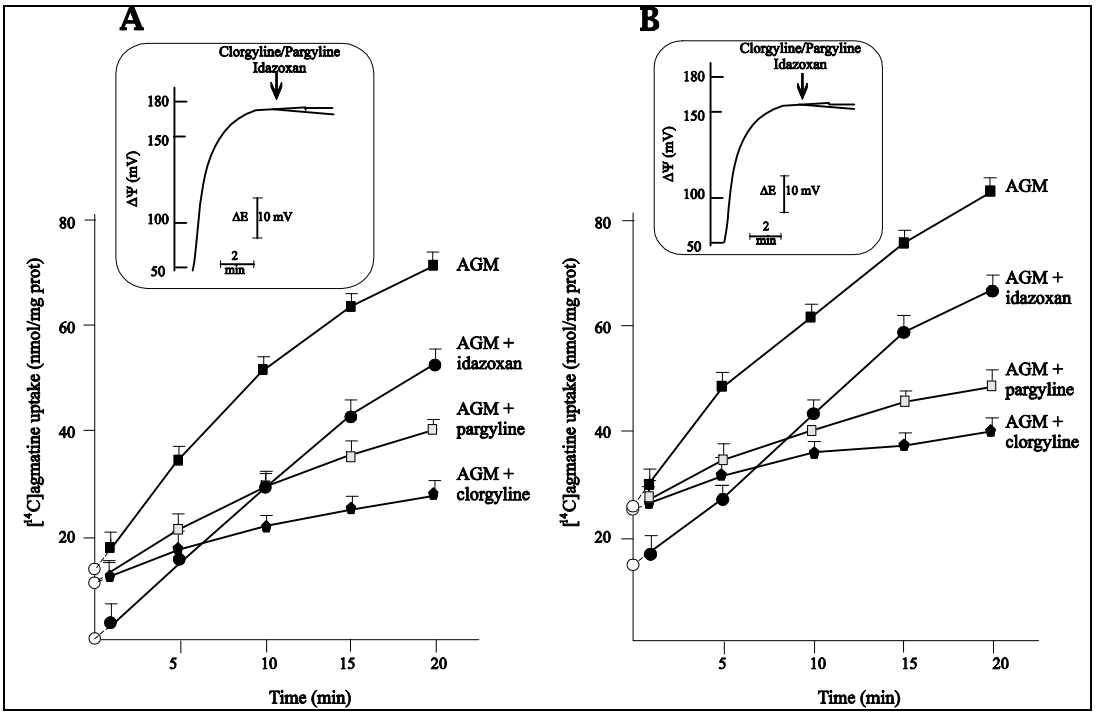
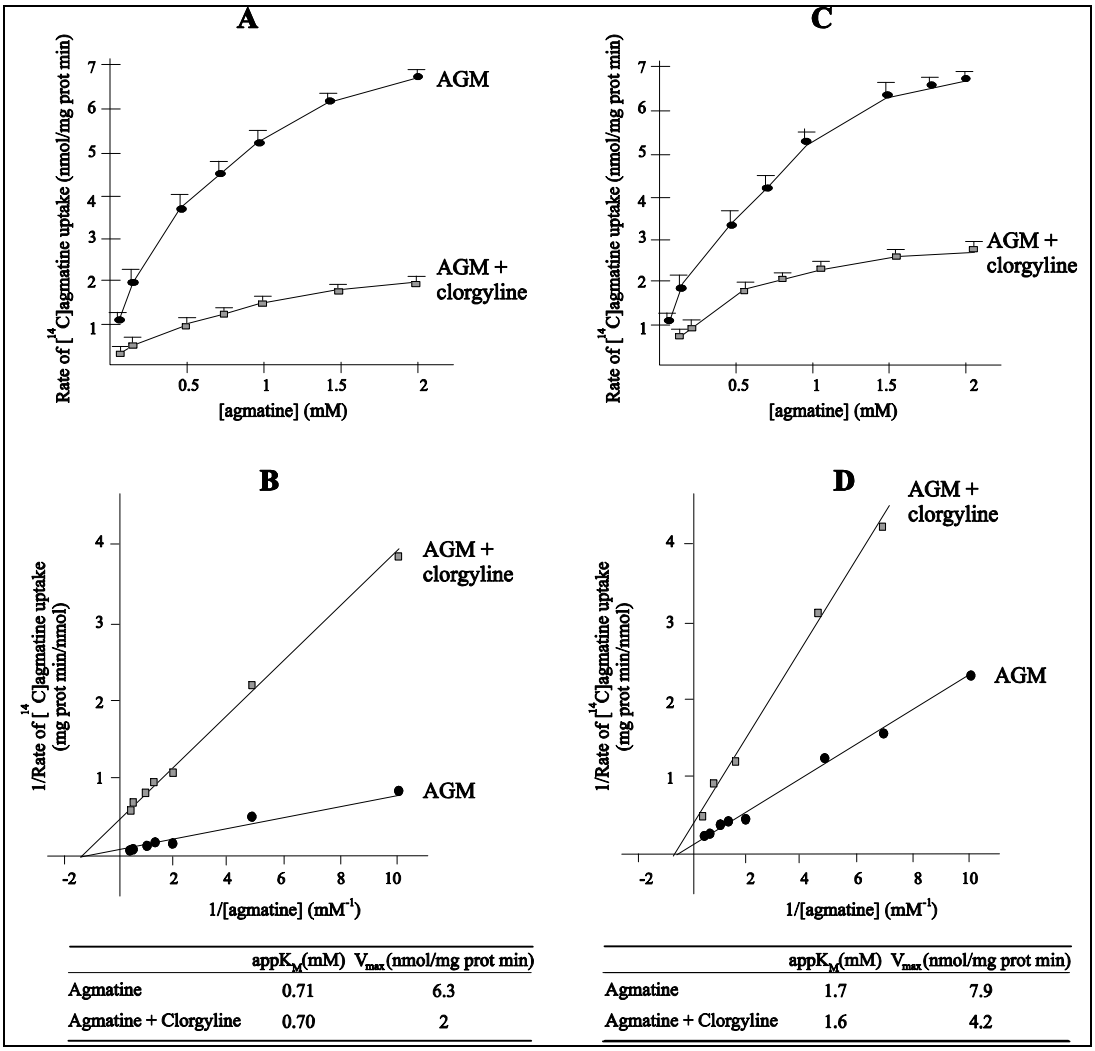


Fig. 7. Effect of idazoxan and propargylamines on agmatine uptake in RLM (A) and RKM (B).
Mitochondria were incubated in standard medium, as described in Materials and Methods section, with 1 mM [^{14}C]agmatine (50 μCi/mmol). When present in the medium: 50 μM clorgyline, 100 μM pargyline and 200 μM idazoxan. Values are the means ±SD of five experiments. Inset: determination of ΔΨ. ΔE=electrode potential.

Transport inhibition is observed in the presence of the propargylamines, clorgyline and pargyline, well known inhibitors of MAO activity (fig. 7). It is to note that these inhibitors do not affect ΔΨ (fig. 7, inset). These propargylamines have a single protonated amino group [De Marchi et al., 2003], so that their inhibition sustains the hypothesis that agmatine is transported as a monovalent rather than a divalent cation.

As agmatine is able to bind to the I_2 imidazoline receptor, located on the mitochondrial membrane, the experiment shown in fig. 7 was also performed with the aim of verifying whether this receptor is involved in agmatine transport. The results show that the I_2 inhibitor idazoxan does not prevent its net transport but completely inhibits the initial membrane binding of agmatine (see the extrapolation of transport traces at zero time, indicated by the empty circles, fig. 7).

The observation that propargylamines inhibit agmatine transport with no significant inhibition of initial binding and that idazoxan behaves in the opposite way, indicates that there is more than one binding site for agmatine on the mitochondrial membrane.



	appK$_M$(mM)	V$_{max}$(nmol/mg prot min)
Agmatine	0.71	6.3
Agmatine + Clorgyline	0.70	2

	appK$_M$(mM)	V$_{max}$(nmol/mg prot min)
Agmatine	1.7	7.9
Agmatine + Clorgyline	1.6	4.2

Fig. 8. Saturation kinetics and double reciprocal plot of agmatine uptake in RLM and RKM. Inhibitory effect by clorgyline.
A and C: RLM (A) and RKM (C) were incubated for 5 min in standard medium, as described in Materials and Methods section, with [^{14}C]agmatine (50 μCi/mmol) at the indicate concentrations. When present, clorgyline was 1 mM. The uptake of agmatine was linear over the incubation period. Values are the means ±SD of five experiments. B and D: Double reciprocal plot of the data shown in A and C, respectively. Inset: appK$_M$ and V$_{max}$ calculated by computer simulation.

Agmatine transport exhibits saturation kinetics (fig. 8, panels A and C) and the calculated kinetic parameters for RLM gives appK$_M$ of 0.71 mM and V$_{max}$ of 6.32 nmol/min · mg protein, whereas for kidney are appK$_M$ of 1.7 mM and Vmax of 7.9 nmol/min · mg protein. These parameters are similar to that of polyamines (e.g. appK$_M$ and V$_{max}$ of putrescine transport are 1 mM and 1.14 nmol/min · mg protein, respectively [Toninello et al., 1992]), suggesting that the transporter of agmatine might be the same as that of polyamines.

The kinetic parameters are calculated also in the presence of 50 μM clorgyline to identify the type of inhibition induced by the

propargylamines. The results of fig. 5 show that clorgyline inhibits the initial rate of agmatine transport in a non-competitive manner, as demonstrated by the double reciprocal plot (panels B and D). In this case the appK_M is 0.70 mM and the V_{max} is 2 nmol/min · mg protein for RLM and 1.6 mM and 4.2 nmol/min · mg protein for RKM.

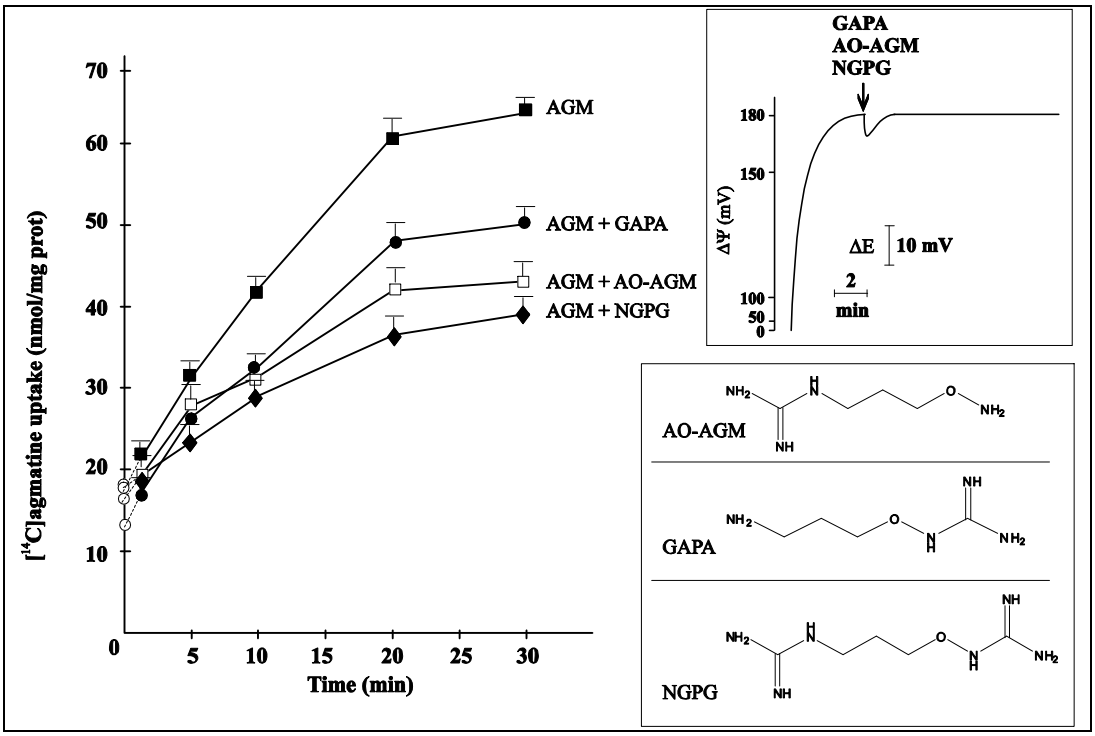


Fig. 9. Transport of agmatine in RLM in presence of analogues.
RLM were incubated in standard medium, as described in Materials and Methods section, with [^{14}C]agmatine (50 μCi/mmol). When present, GAPA, AO-AGM and NGPG are 1 mM. Values are the means ±SD of five experiments. Inset: effect of analogues on ΔΨ.

The observation that arginine, having the same guanidine group as agmatine, does not inhibit agmatine transport in RLM (fig. 6), leads to investigate if other guanidine compounds are equally ineffective. These compounds are provided by the lab of Dr. Khomutov (Russia), and are named: AO-AGM [N-(3-aminooxypropyl)-guanidine], GAPA [N-(3-aminopropoxy)-guanidine] and NGPG [N-(3-guanidino-propoxy)-guanidine] (see inset in fig. 9 for structures) [Simonyan et al., 2005]. The three compounds inhibit the transport with different efficacy and cause a little reduction in the instantaneous binding of agmatine to RLM (fig. 9). The compounds do not alter ΔΨ values, so the analogues most probably are transported electrophoretically via the agmatine transporter.

Characterization of agmatine transport in RBM

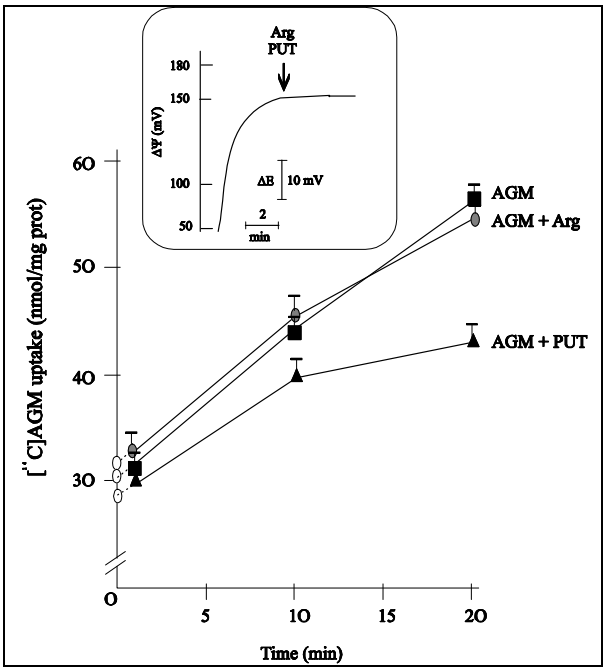


Fig. 10. Effect of polyamine and amino acids on agmatine uptake in RBM.
RBM were incubated in standard medium, as described in Materials and Methods section, with 1 mM [^{14}C]agmatine (50 μCi/mmol). When present in the medium: 1 mM putrescine (PUT), 1 mM arginine (Arg). Empty circles on the ordinate axis indicate agmatine bound at zero-time. Values are the means ±SD of five experiments. Inset: determination of ΔΨ. ΔE=electrode potential.

The transport of agmatine, is not shared with arginine, also in RBM, in fact, the results in fig. 10 demonstrate that the amino acid does not inhibit the uptake of the amine. On the contrary, putrescine inhibits agmatine transport. Thus, we can hypothesize that the transporter of agmatine in RBM is the same of the polyamines.

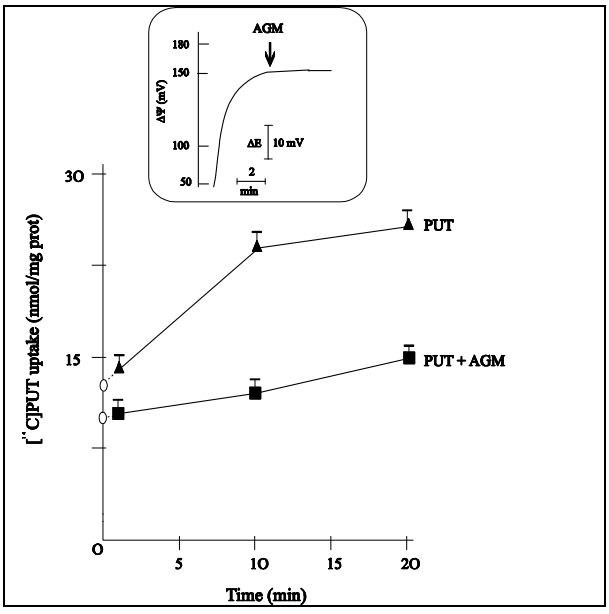


Fig. 11. Putrescine uptake by RBM.
RBM were incubated in standard medium, as described in Materials and Methods section, with 1 mM [^{14}C]putrescine (50μCi/mmol). When present: 1 mM agmatine (AGM). Dotted lines and empty circles on ordinate axis indicate the extrapolation of agmatine binding at zero-time. Inset: determination of ΔΨ. Values are the means ±SD of five experiments.

To test this hypothesis I measured [^{14}C]putrescine transport in the presence of cold agmatine. The presence of this diamine inhibits the

putrescine uptake in RBM, so it is possible to suggest that the transporter is common for agmatine and polyamines.

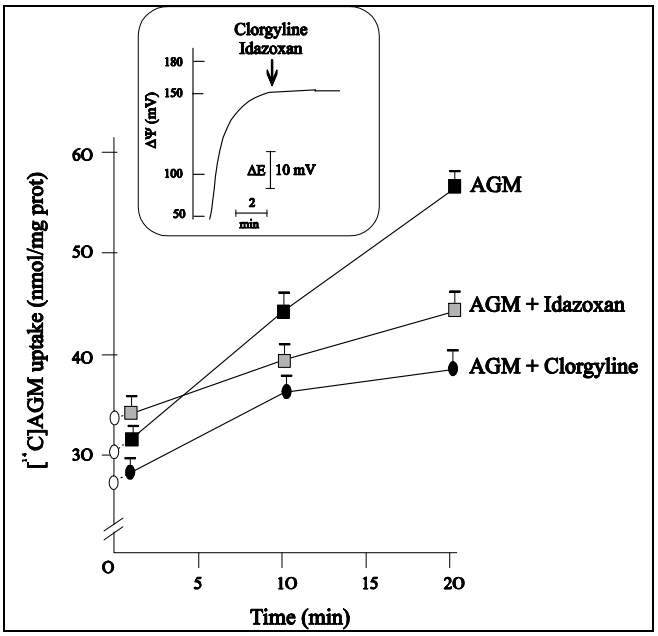


Fig. 12. Effect of idazoxan and clorgyline on agmatine uptake in RBM.
RBM were incubated in standard medium, as described in Materials and Methods section, with 1 mM [^{14}C]agmatine (50μCi/mmol). When present in the medium: 50 μM clorgyline and 200 μM idazoxan. Values are the means ±SD of five experiments. Inset: determination of ΔΨ. ΔE=electrode potential.

Clorgyline inhibits the net transport of agmatine without affecting the initial binding as in RLM and RKM (compare fig. 12 with fig. 7). The I_2 imidazoline receptor inhibitor, idazoxan, exhibits a different behavior than in other mitochondria, in fact it does not affect the initial binding but inhibits the net transport. This observation suggests that, in RBM, an involvement of imidazoline receptors in agmatine transport can occur.

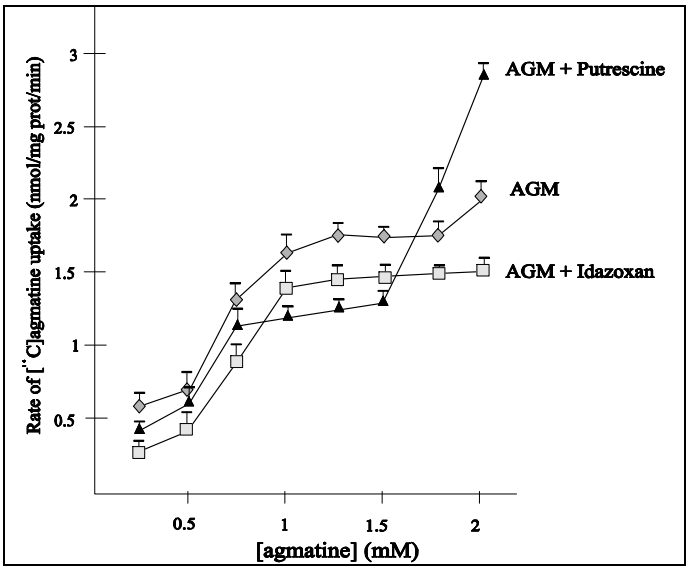


Fig. 13. Saturation kinetic of agmatine uptake in RBM.
RBM were incubated for 5 min in standard medium, as described in Materials and Methods section, with [^{14}C]agmatine (50μCi/mmol) at the indicate concentrations. When present: 1 mM putrescine and 200 μM idazoxan. The uptake of agmatine was linear over the incubation period. Values are the means ±SD of three experiments.

The results in fig. 13 demonstrate that agmatine transport in RBM exhibits a kinetics with a S curve, similar but more complex to the activity of an allosteric enzyme. This seems to evidence a possible

cooperative effect of the agmatine transporter and probably the presence of more than one transport sites. The increase in the rate of agmatine uptake at concentrations >1.5 mM, with or without putrescine, leads to hypothesize that could be there also more than one regulatory sites in the transporter. So, the inhibitors can act in both type of sites, involved in transport and its regulation.

Moreover, the addition of putrescine or idazoxan, which inhibit the uptake of agmatine (figs. 6, 7), leads to the conclusion that the polyamines transporter and imidazoline I_2 receptor are involved in the agmatine transport, but the particular kinetics does not permit to calculate precisely the type of inhibition. Experiments are in progress in order to better elucidate this transport kinetics.

The computer calculation of kinetic parameters is performed considering the Hill graph for allosteric enzymes. Thus, the app$K_{0.5}$ is of about 0.67 mM and the corresponding V_{max} of 1.7 nmol/min · mg prot.

Discussion

Although no specific agmatine transport mechanism in cells has, to this date, been characterized at a molecular level, several proposed models are reported in the literature. One of these (e.g., in human cell lines derived from embryonic kidney) suggests that agmatine may be transported through the EMT or the OCT2 [Grundemann et al., 2003]. In this particular case, it was verified that the transport velocity is directly proportional to the concentration of the monopositive form of the molecule. At physiological pH agmatine is considered a divalent cation. Nevertheless, due to the presence of an alkaline microenvironment inside the agmatine transporter, this amine, most probably, should be transported as a monovalent cation instead as dipositive species [Toninello et al., 2006]. This is supported by the high dipole moment of the monovalent agmatine, as respect to that of polyamines, and by the observation that the initial rate of agmatine transport is higher than that of polyamines [Toninello et al., 1992]. Moreover, the inhibition of agmatine transport by propargylamines (fig. 7), which have a single protonated amino group, sustains the above hypothesis that agmatine is transported as a monovalent cation.

I reported the evidence that agmatine is capable to binding at mitochondrial membranes and is taken up into the matrix space of mitochondria. This binding is most probably electrostatic in nature and is affected by natural polyamines (figs. 6, 10) and idazoxan (fig. 7), and is unaffected by de-energizing agents (fig. 5) as well as cationic amino acids (fig. 6, 10). Agmatine binding is followed by uptake which is highly dependent on mitochondrial energization and is electrophoretic in nature (fig. 5).

As above mentioned, the polyamine transporter is common to all natural polyamines, so that they reciprocally inhibit their transport in a competitive manner [Toninello et al., 1992]. In RLM and RKM the addition of putrescine does not inhibit agmatine uptake, by indicating the existence of different transport systems for agmatine and polyamines (fig. 6).

In mitochondria isolated from all three organs, liver, kidney and brain, the transport is not inhibited by the addition of arginine (figs. 6, 10), thus excluding the possibility that agmatine can use the electroneutral transport of basic amino acids.

Strong inhibition of agmatine transport is observed with clorgyline and pargyline (figs. 7, 12). These propargylamines act as non-competitive inhibitors of transport and function, independently of action on MAO (fig. 8). Observations that some compounds, e.g. putrescine (fig. 6) and idazoxan (fig. 7), decrease initial binding

without affecting transport, in both RLM and RKM, whereas other, such as propargylamines, inhibit transport without inhibiting the initial binding, indicate that there are at least two types of binding sites for agmatine on mitochondrial membranes. These two binding sites (S_1 and S_2) exhibit mono-coordination, with high binding-capacity and low-binding affinity [Salvi et al., 2006], as also observed for polyamines [Dalla Via et al., 1996 and 1999]. The dissociation costants of both sites demonstrate that the binding affinity of S_1 is approx. 200-fold higher than that of S_2 [Salvi et al., 2006]. Since previous investigations on polyamine transporter have shown that the site with the higher affinity is linked to the transport [Dalla Via et al., 1999], S_1 is evidently responsible for the transport of agmatine. The non-competitive inhibition of clorgyline in this transport (fig. 8) excludes the possibility that both molecules are taken up by the same transporter, and the incomplete inhibition is consistent with a residual binding of agmatine to its transporter (S_1 site). Idazoxan, instead, inhibits only the initial binding (fig. 7) and, most likely, interacts only with the S_2 site.

Flux-voltage analysis have been performed to understand the type of transporter [Salvi et al., 2006]. The energy barriers calculated for agmatine transport lead to the conclusion that the amine in divalent form is transported by a uniport that may be a channel, similar to that of polyamines [Toninello et al., 1992]. However, calculation for the monovalent agmatine, which, as above mentioned, is most probably present in the microenvironment of the transporter and is the main transported form, demonstrates that the amine is taken up by a single-binding centre-gated pore, of which a typical example is the ATP/ADP carrier [Huang et al., 2001].

Then, the transport mechanism of agmatine in RLM and RKM is very similar and involves, probably, a channel or a single-gated centre-gated pore specific for the amine [Salvi et al., 2006]. Moreover, the $appK_M$ of the uptake in RLM and RKM (0.71 and 1.7 mM, respectively, fig. 8) are compatible with the concentration of the amine in liver and kidney (>0.5 mM), and also with the observed variations in agmatine concentration in some pathological conditions [Galea et al., 1996].

To better understand the origin of agmatine transporter, I have performed also experiments on the amine transport in RLM, in the presence of the new, recently synthesized, charge-deficient agmatine analogues: AO-AGM, GAPA and NGPG. These compounds are synthesized to study the chemical regulation of polyamine metabolism [Simonyan et al., 2005]. The results of fig. 9 demonstrate that all the three compounds inhibit the agmatine transport in RLM. Kinetic studies on this inhibition show that AO-AGM and NGPG act as competitive inhibitors, whereas GAPA is non-competitive [Grillo et al.,

2007]. Thus, the guanidine group is of primary importance. In fact, it is the group of the inhibitors which competes with that of agmatine in binding to the transport site. The explanation for the lack of the inhibition of arginine in the agmatine transport (fig.6) is probably due to the fact that the carboxy group of the amino acid hampers its binding to the transporter.

In RBM, instead, the transporter of agmatine has some difference with that of other mitochondria. First of all the inhibition exhibited by putrescine (fig. 10), which could signify that the transport of agmatine is shared with the polyamines. To verify this hypothesis I evaluated also the transport of putrescine, in the presence of agmatine which results to be inhibited. This confirms the possibility of a unique transporter for these amines (fig. 11). Moreover, it has been observed an inhibition of transport in the presence of idazoxan, which could mean that the I_2 imidazoline receptor, present on mitochondrial membranes, is involved in agmatine uptake (fig. 10). Considering the inhibition of clorgyline (fig. 12) and the co-localization of I_2 receptors and MAO [Tesson et al., 1995], it is possible that they are involved in agmatine uptake in RBM.

Kinetic analyses of agmatine uptake in RBM demonstrate that the agmatine transporter, in these mitochondria, could be similar to an oligomeric protein having positive coordination (fig. 13), so more than one transport site is present and, probably, more than one subunity constitute the transporter. The cooperative effect exhibited by increasing agmatine concentration is suggested by the apparent S curve (fig. 13). At concentrations over than 1.5 mM, a further increase in the rate of agmatine transport is observable, this leads to hypothesize the presence of more regulatory sites in the agmatine transporter. In the presence of idazoxan this increase does not take place by suggesting that the I_2 receptor exhibits a regulatory role on agmatine transport. In the presence of putrescine, instead, the increase is further amplified, so, probably, the contemporaneous presence of agmatine and polyamines provokes an increase in the affinity of the transporter with a positive cooperativity between the two types of molecules. Other studies to understand the real nature of this transporter and the calculation of the kinetic parameters are now still in progress.

2. Action of agmatine in mitochondrial permeability transition (MPT) induction

The mitochondrial permeability transition

The mitochondrial permeability transition (MPT) is a phenomenon strictly connected with apoptosis induction. This phenomenon takes place in presence of specific inductors and with an altered calcium homeostasis. In this condition, the impermeability of mitochondrial inner membrane, necessary to the establishment of the electrochemical gradient ($\Delta\mu_H^+$), is seriously compromised with a consequent block of ATP synthesis.

The energy production from mitochondria needs a complete impermeability of inner mitochondrial membrane, in which only specific transporters permit the passage of solutes across the membrane, as described in Mitchell's chemi-osmotic model (1961). In the inner membrane, during permeability transition, there is the opening of an aspecific channel at high conductance that permits the transit of solutes having molecular mass less than 1500 Da, the permeability transition pore (PTP). The result of the PTP opening is the collapse of $\Delta\mu_H^+$, mitochondrial swelling and rupture of the outer mitochondrial membrane with the release of some apoptotic factor [Zoratti and Szabò, 1995].

The permeability transition pore

The PTP is a protein complex with a diameter of 2-3 nm which permits a bidirectional traffic of molecules until 1500 Da, as above mentioned. In the formation of the pore different proteins are involved: the adenine nucleotides translocase (AdNT) in the inner membrane, the cyclophilin D (CypD) in the matrix, and the voltage dependent anion channel (VDAC) in the outer membrane. These proteins presumably form the core of the complex. Instead, creatine kinase, hexokinase, benzodiazepine receptor, proteins of Bcl-2 family and others kinases are additional or regulator components [Zoratti et al., 2005]. Very recently, an important role in PTP formation has been ascribed to phosphate (Pi) carrier [Leung et al., 2008].

The AdNT is an electrogenic antiport exchanging endogenous ATP with exogenous ADP [Halestrap, 1987]. Its activity is favored by the transmembrane electrochemical gradient, positive in the outer side of the inner membrane, since the AdNT bring out one negative charge (ATP^{4-} against ADP^{3-}). The AdNT structure is stabilized in the outer binding sites by ADP and in the inner by ATP. These nucleotides, when present, inhibit the pore opening. Some molecules, able to affect AdNT activity, are also regulators of the pore, e.g. bongkrekic acid (BKA) and

actractylate that induce and inhibit, respectively, the MPT [Halestrap and Davidson, 1990]. In a recent study, the involvement of AdNT in the PTP has been proposed to be not essential in the formation of the pore, but the lack of this protein would prevent the regulation of PTP. This suggests that AdNT may have only a regulatory role in controlling PTP induction [Leung et al., 2008].

The CypD is a peptidyl-prolyl-*cis-trans*-isomerase (PPIase) normally located in the mitochondrial matrix. The involvement of this molecule in the PTP formation is demonstrated by the inhibition of cyclosporine A (CsA), a ligand of CypD, on the MPT induction. The PTP opening involves a conformational change in a membrane protein which is facilitated by the PPIase activity of CypD. In CypD knockout mice the MPT happens but in the presence of a very high Ca^{2+} concentrations [Leung and Halestrap, 2008].

The VDAC, also known as porin, interacts with AdNT at contact sites, points of intimate contact between the inner and outer mitochondrial membranes. The other proteins involved in PTP formation interact also in contact sites, e.g. creatine kinase, Bcl-2, Bax and hexokinase. It has been demonstrated that mitochondria lacking of VDAC exhibit normal PTP opening, thus proving that VDAC is not an essential component of the pore [Leung and Halestrap, 2008].

Very recently it has been demonstrated that CypD binds also the Pi carrier in association with AdNT, and thus the Pi carrier could be important for PTP formation [Leung et al., 2008]. The fact that Pi is a potent activator of MPT confirms this hypothesis.

Induction of MPT

The MPT takes place in the presence of supraphysiological Ca^{2+} concentrations, together with an inductor and/or oxidative stress.

Ca^{2+} is transported in mitochondrial matrix by two systems: an electroforetic uniport, specific for the uptake and an electroneutral antiport for the exit. The efflux occurs in exchange with two protons (H^+) or two Na^+ for every Ca^{2+} [Skulachev, 1999]. These tranporters contributes to maintain the calcium homeostasis in cells and, very similar, in endoplasmic reticulum to release Ca^{2+}, when necessary, in the cytosol. During the MPT, instead, Ca^{2+} is released from mitochondria provoking modifications on activity of several mitochondrial enzymes regulated by its concentrations.

One of the most studied inducers of MPT is Pi. Pi crosses the mitochondrial membrane as uncharged ortophosphoric acid (H_3PO_4), it dissociates in matrix and reduces the inner alkaline pH by the release

of $2H^+$. This determines the increases in the $\Delta\Psi$, with consequent increase in the accumulation of Ca^{2+}.

Other inducers of MPT provoke the production of ROS in mitochondria, with consequent alteration of the redox state of several mitochondrial components, as pyridine nucleotides, thiols, glutathione. Examples of this type of inducers are salicylate and glycyrrhetinic acid [Battaglia et al., 2005; Fiore et al., 2004].

The oxidation of pyridine nucleotides is a phenomenon strictly associated with the induction of MPT, but it is not clear if the oxidation takes place before the opening of PTP, thus being a pre-requisite, or if it is only responsible for the amplification. The oxidation of membrane thiols forms disolphure bridges that destabilize the membrane structure and favor the opening of PTP. In this regards it has been proposed that the oxidation of two critical thiols, most probably located on AdNT, is responsible of pore opening [Leung and Halestrap, 2008].

All the effects on redox status can induce the MPT but also can be a consequence of it. Once the PTP is open $\Delta\Psi$ collapses and the rate of respiration increases, this causes production of ROS which provokes a further oxidation in the above components.

Inhibition of MPT

The inhibitors of MPT are molecules that interfere with Ca^{2+} accumulation, by the action of the inducers, or act on the structural components of the PTP by blocking its opening.

At the last category belongs the immunosuppressant CsA. It binds CypD, thus preventing its interactions with the pore which remains in the closed conformation [Crompton et al., 1988]. Other inhibitors act on AdNT to maintain its physiological conformation: ADP, ATP and BKA. Moreover BKA acts also to maintain high the adenine nucleotide concentration [Zoratti and Szabò, 1995].

The inhibitors of Ca^{2+} accumulation include ruthenium red, which inhibits the entry by inhibiting the uniport, and chelating agents (e.g. EGTA) which binds Ca^{2+} in solution.

Finally, among the inhibitors of MPT, the reducing agents and scavengers of ROS are to include. Spermine, for example, exhibits a protective effects against the MPT by its action of scavenger [Sava et al., 2006].

Model of PTP formation

The typical model for the PTP opening induced by Ca^{2+} and Pi has been proposed by Halestrap and Davidson (1990) and involves the AdNT. In this model, Pi and pyrophosphate, produced by ATP hydrolysis, catalyzed by a Ca^{2+}-dependent pyrophosphatase, bind to the ADP and ATP binding sites, respectively. The AdNT becomes a potassium channel CsA-insensitive. At this point, Ca^{2+} binds in a inner site of AdNT and a conformational change permits the interaction of CypD with the AdNT which becomes the CsA-sensitive PTP.

According to other authors [Kim et al., 2003], this "regulated" opening of PTP is opposed to an "unregulated" opening that is determined to an incorrect folding and aggregation of some membrane proteins in presence of MPT inducers, mainly by oxidant agents. These misfolded proteins forms aqueous channels that permit the passage of molecules at low molecular weight. CypD normally blocks the conductance through this type of channel by acting as a chaperon to catalyze the correct folding. Thus, the binding of CypD makes the "regulated" PTP. When misfolded protein clusters exceed the CypD available to block conductance, "unregulated" PTP opening occurs. The apparent involvement of AdNT in PTP formation is explained in terms of the high amount of this protein in the inner mitochondrial membrane.

A recent model proposes that the main pore-forming component is the Pi carrier rather than the AdNT. This transporter would undergo a Ca^{2+}-triggered conformational change in order to induce PTP formation which is facilitated by the isomerase activity of CypD. An interaction of the Pi carrier with the "c" conformation of AdNT (induced by actractylate which sensitizes pore opening to Ca^{2+} concentration) is proposed to enhance the sensitivity of the Pi carrier to the conformational change able to induce PTP opening. On the contrary, the "m" conformation of AdNT (induced by BKA which inhibits the PTP opening) exerts no effect or inhibits the process [Leung and Halestrap, 2008].

Another group proposes that Pi has a desensitizing effect on the PTP [Basso et al., 2008]. Pi is able to bind a regulatory site which is not accessible when CypD is present. Thus, the presence of CypD prevents the inhibitory action of Pi, indicating that the Pi carrier could not be involved in PTP formation.

Effects of MPT induction

The MPT is a key event in cell death induction. The MPT causes osmotic swelling of the mitochondrial matrix, mitochondrial uncoupling, and rupture of the outer membrane with release of the pro-apoptotic factors, e.g. cytochrome c and apoptosis inducing factor

(AIF), in cytosol. Cytochrome c participates in the formation of the apoptosome complex together with its adaptor molecule, Apaf-1, resulting in activation of the caspase cascade in the presence of ATP. AIF, instead, directly activates the endonucleases responsible of DNA cleavage. This model of mitochondrial outer membrane permeabilization may be the most relevant during ischemia/reperfusion injury, or in response to cytotoxic stimuli resulting in localized mitochondrial Ca^{2+} overload [Zoratti et al., 2005].

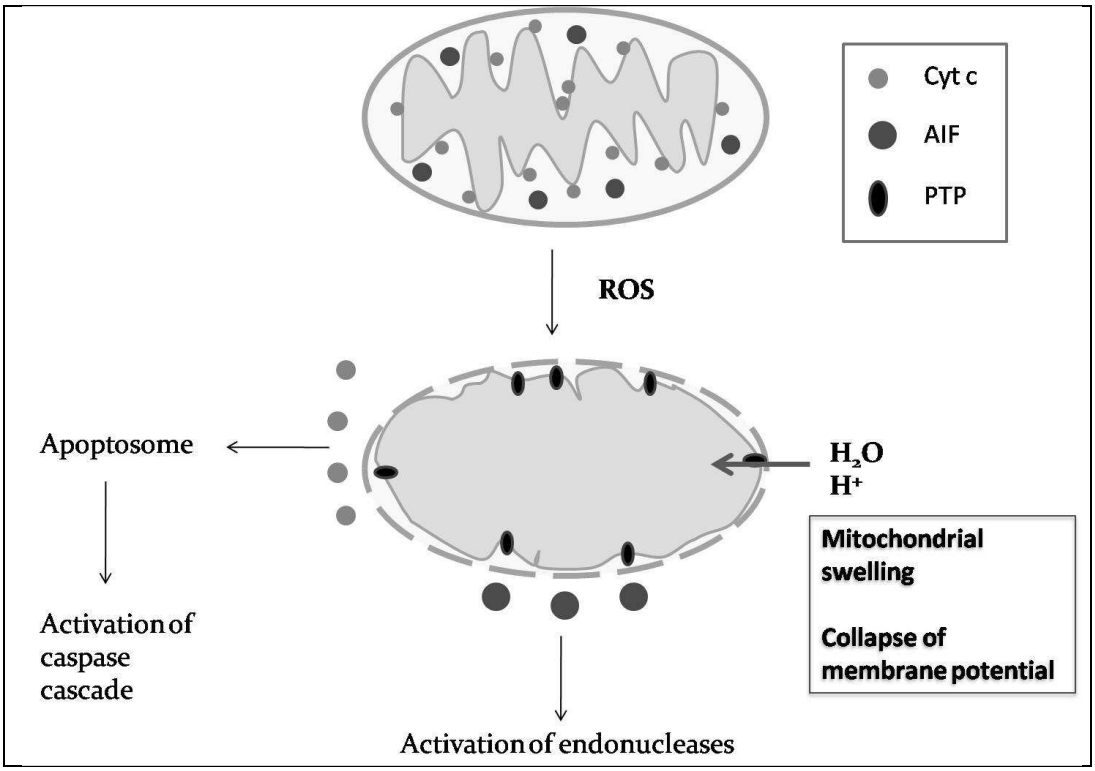


Fig. 14. Release of pro-apoptotic factors during MPT induction.

Since apoptosis requires high ATP content in cell, a profound ATP depletion inhibits apoptotic signaling while simultaneously a necrotic cell death initiates. After the MPT induction, ATP availability from glycolysis and other sources determines whether cell injury progresses to ATP depletion-dependent necrosis or ATP-requiring apoptosis. Thus, apoptosis and necrosis can share a common pathway, the MPT [Lemasters, 2007].

Results

Agmatine concentration reached in blood and tissues (3.5 nM-1 μM) is lower than that able to induce its effects (1-1000 μM), but most likely these levels can be reached in the proximity of its site of action [Molderings et al., 2002]. Moreover, variations in agmatine concentration were observed in pathological conditions (e.g. ischemia) [Del Barre et al., 1995]. The experiments reported in this thesis take into account this possibility and are performed using a physiological range of concentration for agmatine.

Agmatine and mitochondrial bioenergetic functions

Mitochondrial functions are strictly connected with the integrity of the mitochondrial membrane (particularly the inner one) necessary to maintain its insulating properties and a correct electron flux along the respiratory complexes, essential events to establish the electrochemical gradient, characteristic of energy transducing membranes. In order to evaluate agmatine implications on mitochondrial bioenergetic functions it is important to determine its effect on $\Delta\Psi$, the electric component of $\Delta\mu_{H^+}$, and on respiratory control index (RCI), which indicates the coupling between oxygen consumption and ATP synthesis.

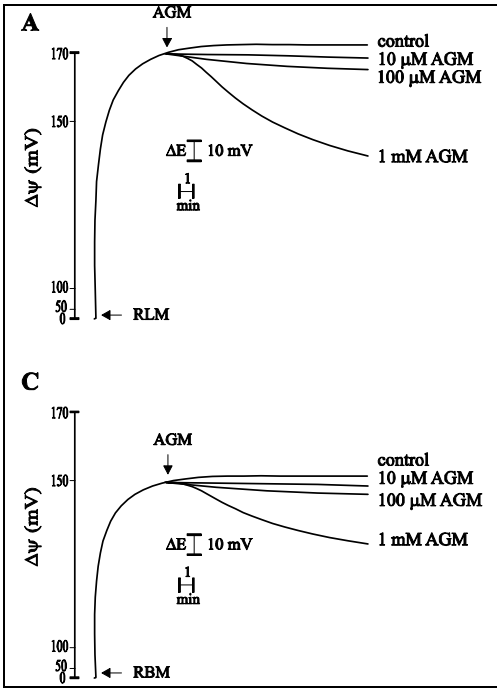
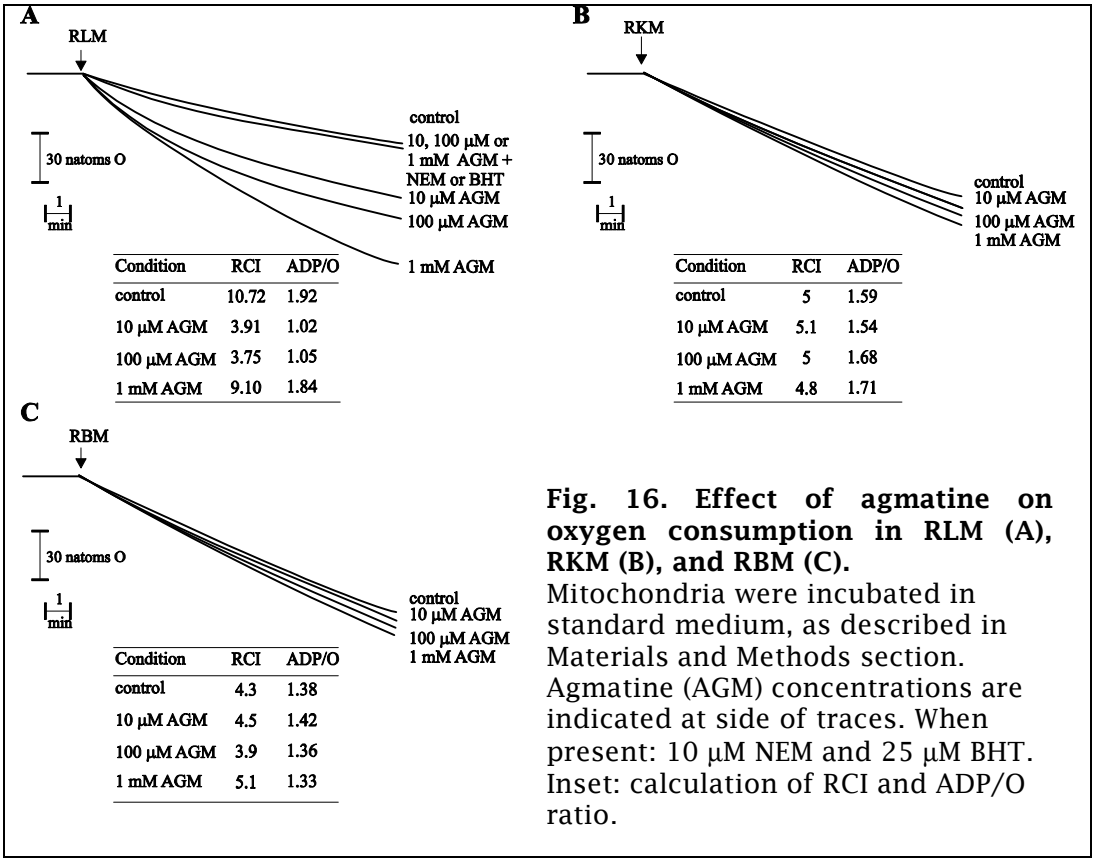


Fig. 15. Effect of agmatine on membrane potential (ΔΨ) in RLM (A), RKM (B), and RBM (C). Mitochondria were incubated in standard medium, as described in Materials and Methods section. Agmatine (AGM) concentrations are indicated at side of traces. ΔE=electrode potential.

Fig. 15 shows the effect of agmatine at three different concentrations (10, 100 μM and 1 mM), on ΔΨ of mitochondria obtained from different organs: liver, kidney and brain. The low concentrations (10 and

100 μM) do not provoke any alterations on ΔΨ in all types of mitochondria. On the contrary, the higher one (1 mM) induces a gradual depolarization, as a result of its transport, in cationic form, in the inner compartment.



Condition	RCI	ADP/O
control	10.72	1.92
10 μM AGM	3.91	1.02
100 μM AGM	3.75	1.05
1 mM AGM	9.10	1.84

Condition	RCI	ADP/O
control	5	1.59
10 μM AGM	5.1	1.54
100 μM AGM	5	1.68
1 mM AGM	4.8	1.71

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

Fig. 16. Effect of agmatine on oxygen consumption in RLM (A), RKM (B), and RBM (C). Mitochondria were incubated in standard medium, as described in Materials and Methods section. Agmatine (AGM) concentrations are indicated at side of traces. When present: 10 μM NEM and 25 μM BHT. Inset: calculation of RCI and ADP/O ratio.

The oxygen consumption increases in the presence of the different concentrations of agmatine according to a dose-dependence in RLM (Fig. 16 A). In the presence of the alkylating N-ethylmaleimide (NEM) or the antioxidant butyl-hydroxytoluene (BHT), the increase in respiration by agmatine is completely abolished. This observation suggests the involvement of ROS generation in the increase of oxygen uptake. In the other two types of mitochondria, RKM and RBM, agmatine, at any concentration tested, does not provoke any alterations in respiration (panel B and C).

The tables in fig. 16 report the calculation of RCI and ADP/O ratio. In RLM (panel A), the low agmatine concentrations reduce both these parameters, indicating that the amine affects ATP biosynthesis by an uncoupling of oxidative phosphorylation. Instead, 1 mM agmatine maintains phosphorylation parameters near normal levels. Once again, RKM and RBM incubated with agmatine do not undergo any significant alterations of these parameters (panel B and C).

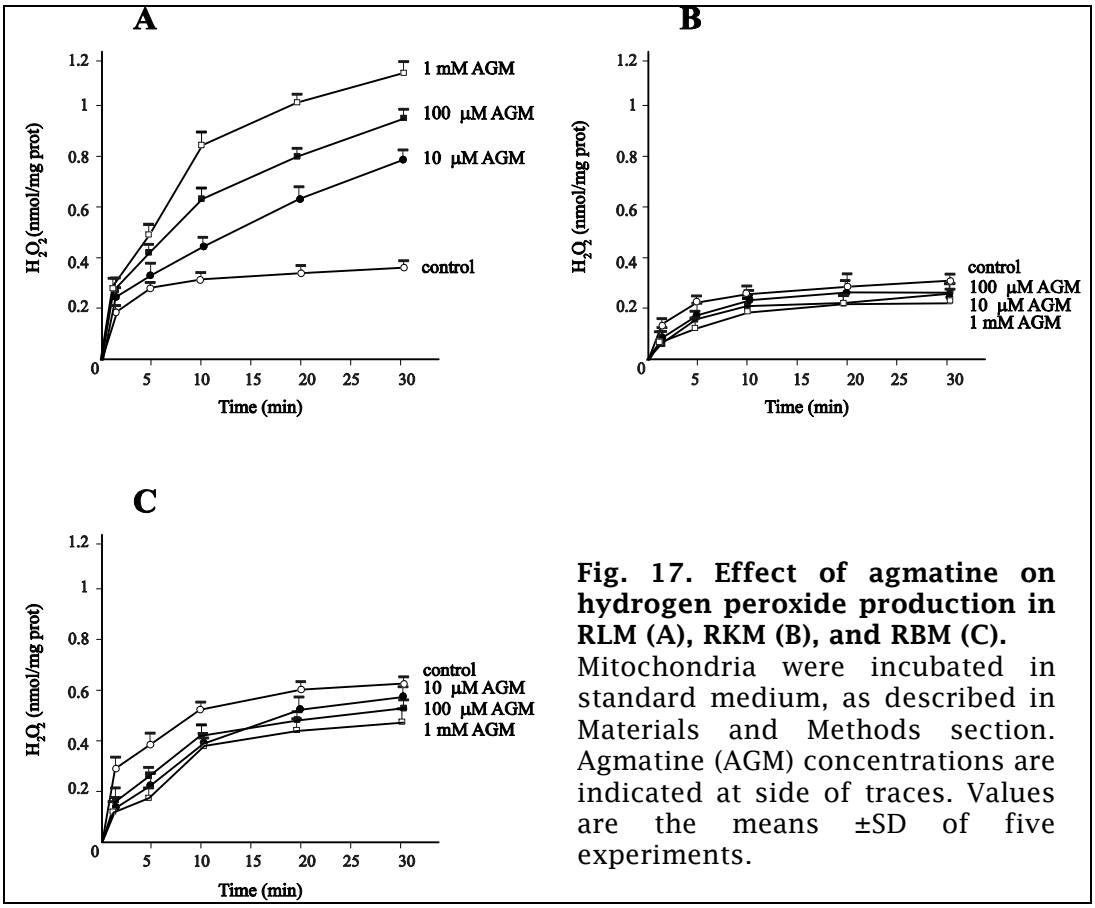


Fig. 17. Effect of agmatine on hydrogen peroxide production in RLM (A), RKM (B), and RBM (C). Mitochondria were incubated in standard medium, as described in Materials and Methods section. Agmatine (AGM) concentrations are indicated at side of traces. Values are the means ±SD of five experiments.

Agmatine provokes a dose-dependent increase in the generation of H_2O_2 in RLM (fig. 17 A), confirming the above hypothesis. The production of ROS by RLM generally leads to oxidative stress, which particularly affects the redox levels of sulfhydryl groups and the $NAD(P)^+$/NAD(P)H pool.

Also in this case, the behavior of agmatine in both RKM and RBM is different to that in RLM (panels B and C). In these types of mitochondria the three concentrations of agmatine decrease the hydrogen peroxide produced by normal mitochondrial functions.

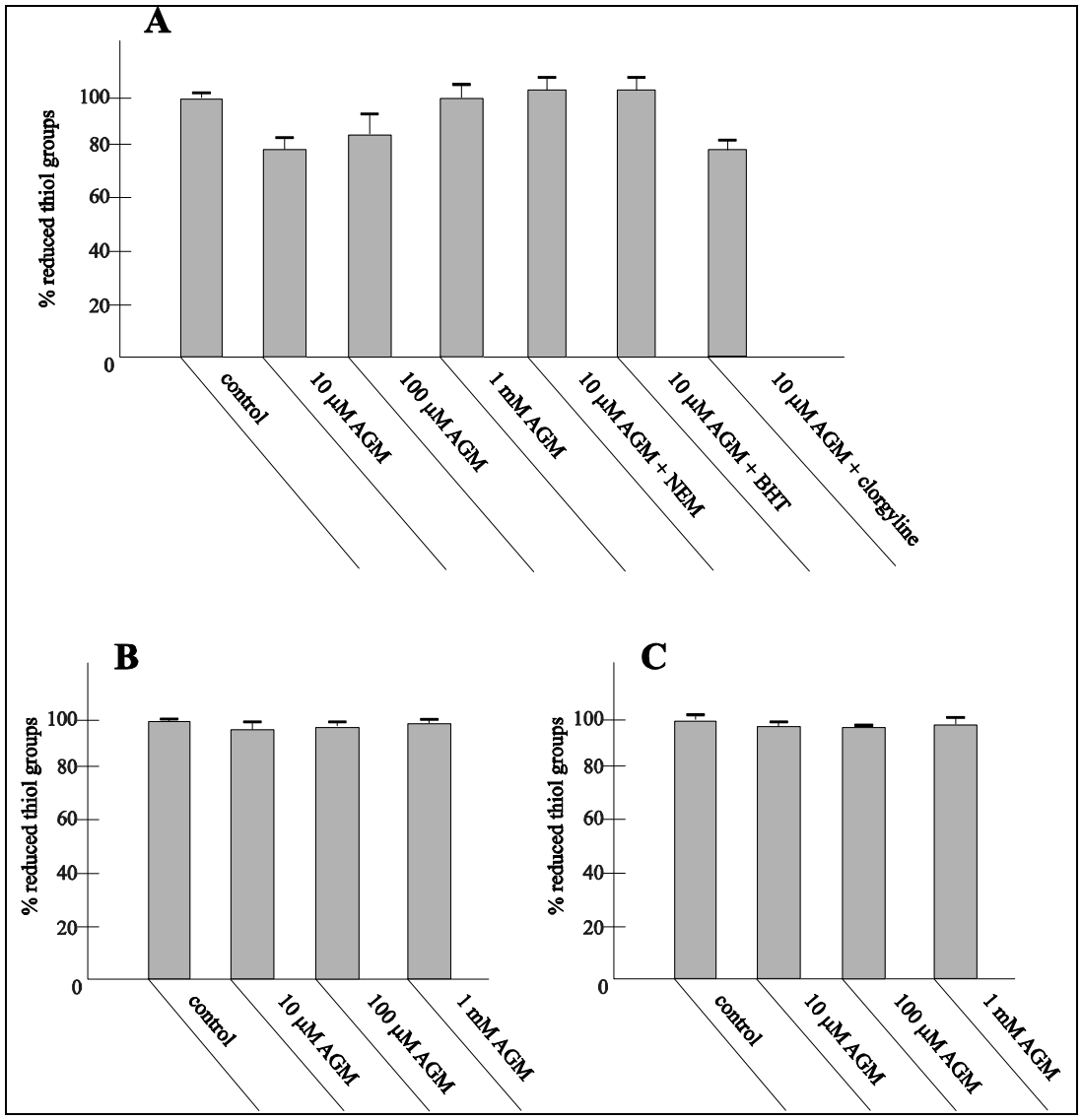


Fig. 18. Effect of agmatine on redox state of sulfhydryl groups in RLM (A), RKM (B), and RBM (C).
Mitochondria were incubated in standard medium, as described in Materials and Methods section. Agmatine (AGM) concentrations are indicated in the histogram. When present: 10 μM NEM, 25 μM BHT and 50 μM clorgyline. Values are the means ±SD of five experiments.

As shown in figure 18 A, 10 μM or 100 μM agmatine induce in RLM a decrease in the content of reduced thiol groups, whereas 1 mM agmatine is almost ineffective. Obviously, a decrease in the reduced thiol groups correspond to an increase in the oxidized ones. Addition of NEM, BHT or clorgyline counteracts the oxidant effects of 10 μM agmatine.

Once again, agmatine in RKM and RBM (panels B and C) does not provoke any alteration in redox state of sulfhydryl groups.

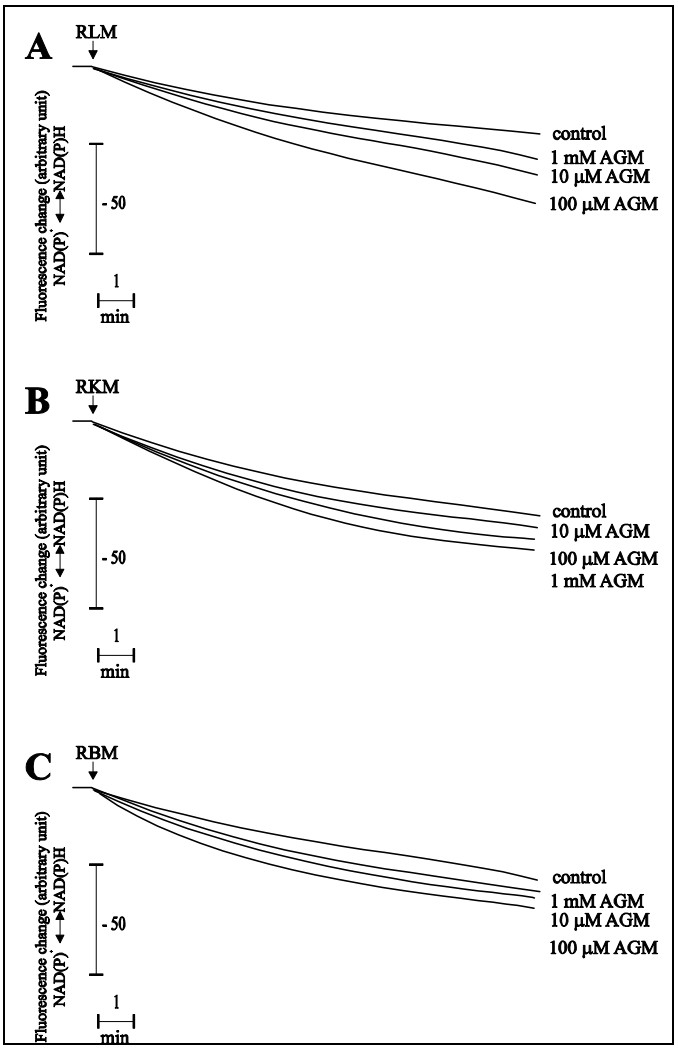


Fig. 19. Effect of agmatine on redox state of pyridine nucleotides in RLM (A), RKM (B), and RBM (C). Mitochondria were incubated in standard medium, as described in Materials and Methods section. Agmatine (AGM) concentrations are indicated at the side of the curves. Values are the means ±SD of five experiments.

When compared with the controls, the results of Fig.19 do not show any appreciable increase by agmatine, at any concentration, in the oxidation of pyridine nucleotides in the different mitochondrial types. This result demonstrates a lack of correlation between redox state variations of thiols and pyridine nucleotides.

Effect of agmatine on MPT

The observation that agmatine, at low concentrations, provokes oxidative effects at the level of important molecular structures in RLM, stimulated the interest to investigate about the physiopathological implication of these effects. First of all the induction of swelling in the presence of Ca^{2+}, an event which correlates MPT with the triggering of apoptosis in cells [Zoratti and Szabò, 1995].

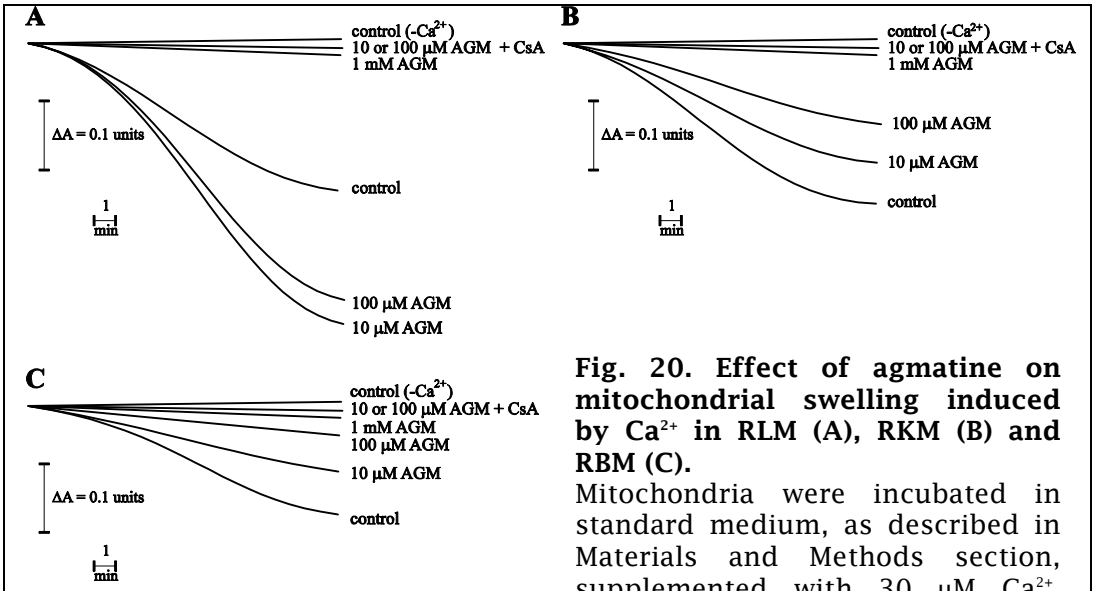


Fig. 20. Effect of agmatine on mitochondrial swelling induced by Ca^{2+} in RLM (A), RKM (B) and RBM (C).
Mitochondria were incubated in standard medium, as described in Materials and Methods section, supplemented with 30 µM Ca^{2+}. When present: 1 µM CsA. Agmatine (AGM) concentrations are indicated at side of the traces.

When suspended in standard medium, in the presence of supraphysiological Ca^{2+} concentrations, mitochondria undergo the phenomenon of MPT, revealed by colloid-osmotic swelling of the inner compartment, detectable by a decrease in the apparent absorbance at 540 nm of the suspension (fig. 20). In RLM, agmatine, at 10 µM or 100 µM, further amplifies absorbance decrease due to Ca^{2+}, which is completely abolished by the MPT inhibitor CsA; at 1 mM agmatine concentration, the phenomenon is completely inhibited (panel A).

At the contrary, in both RKM and RBM (panels B and C), agmatine administration protects, in a dose-dependent manner, against Ca^{2+}-induced mitochondrial swelling.

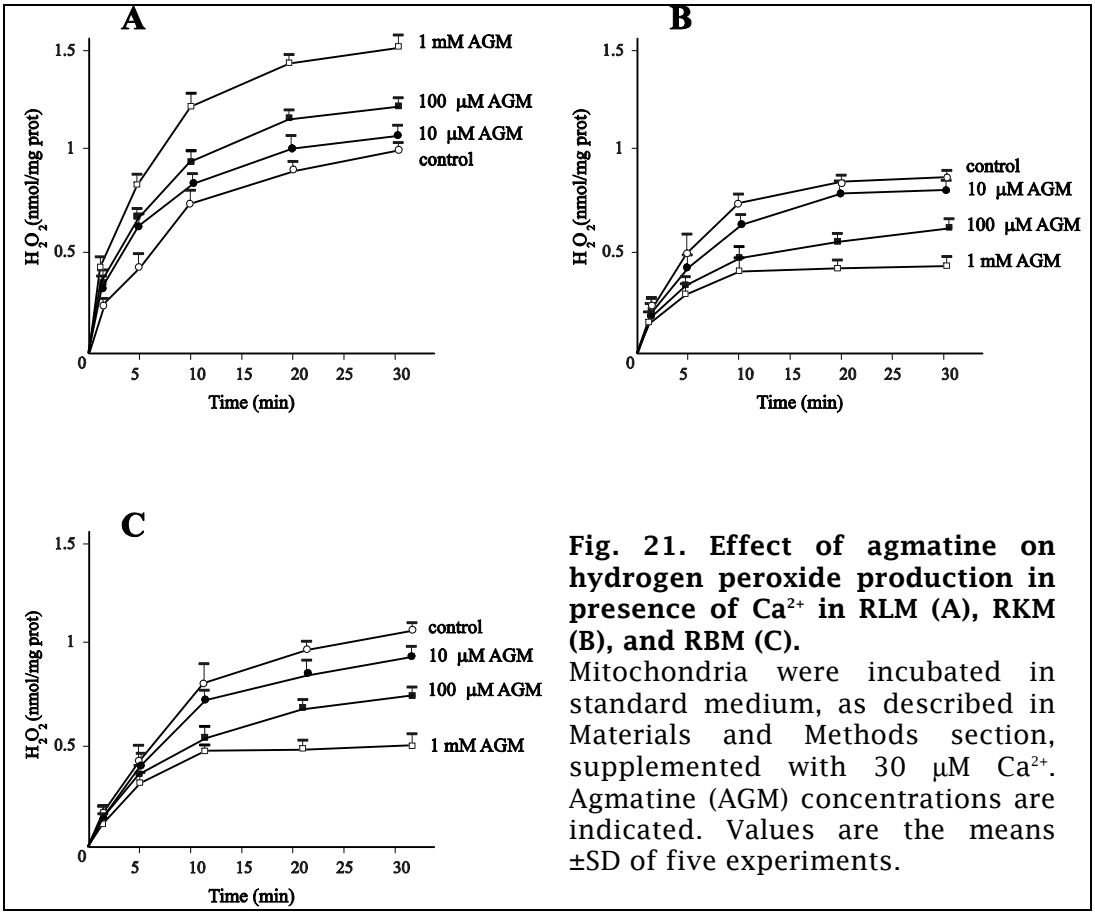


Fig. 21. Effect of agmatine on hydrogen peroxide production in presence of Ca^{2+} in RLM (A), RKM (B), and RBM (C).
Mitochondria were incubated in standard medium, as described in Materials and Methods section, supplemented with 30 µM Ca^{2+}. Agmatine (AGM) concentrations are indicated. Values are the means ±SD of five experiments.

The opening of the PTP by Ca^{2+} is related to production of hydrogen peroxide and alteration of the redox state of different mitochondrial components.

Agmatine, in RLM, provokes a dose-dependent increase in the generation of H_2O_2 particularly in the presence of Ca^{2+} (fig. 21 A). Also in this case, agmatine in RBM and RKM exhibits a dose-dependent protection on the hydrogen peroxide production (panels B and C).

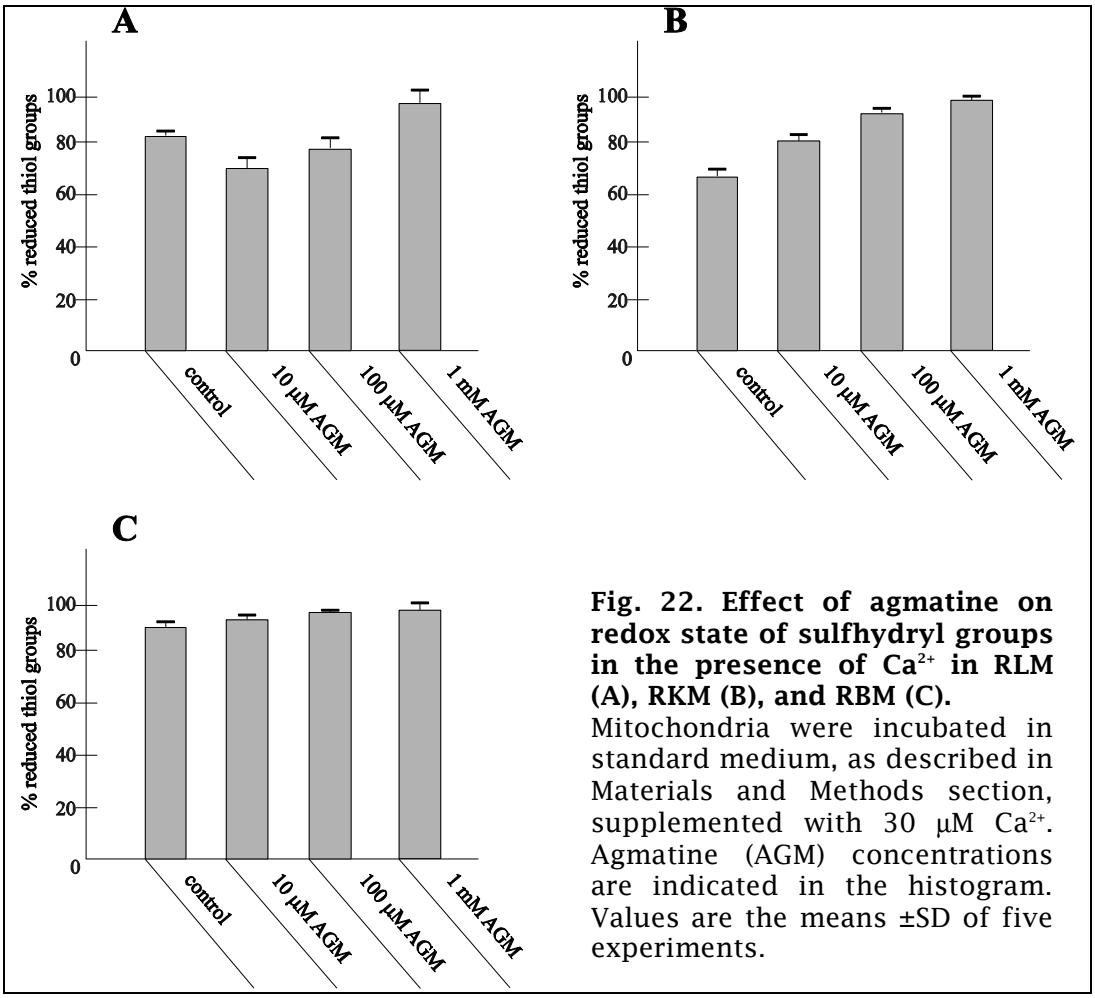


Fig. 22. Effect of agmatine on redox state of sulfhydryl groups in the presence of Ca^{2+} in RLM (A), RKM (B), and RBM (C).
Mitochondria were incubated in standard medium, as described in Materials and Methods section, supplemented with 30 μM Ca^{2+}. Agmatine (AGM) concentrations are indicated in the histogram. Values are the means ±SD of five experiments.

The oxidation of thiols in RLM, in the presence of Ca^{2+} (about 20%), is amplified by 10 μM agmatine (fig. 22 A) which provokes a further increase in thiol oxidation (about 30%) when compared with that of Ca^{2+} or agmatine alone, as shown in fig. 18 A. Instead, 1 mM agmatine maintains thiol redox levels like those of control without Ca^{2+}, that corresponds to the 100% of reduced thiol groups (compare with fig. 18).

In RKM and RBM (panels B and C) the oxidation of thiols induced by Ca^{2+} is protected once again in a dose-dependent manner by agmatine.

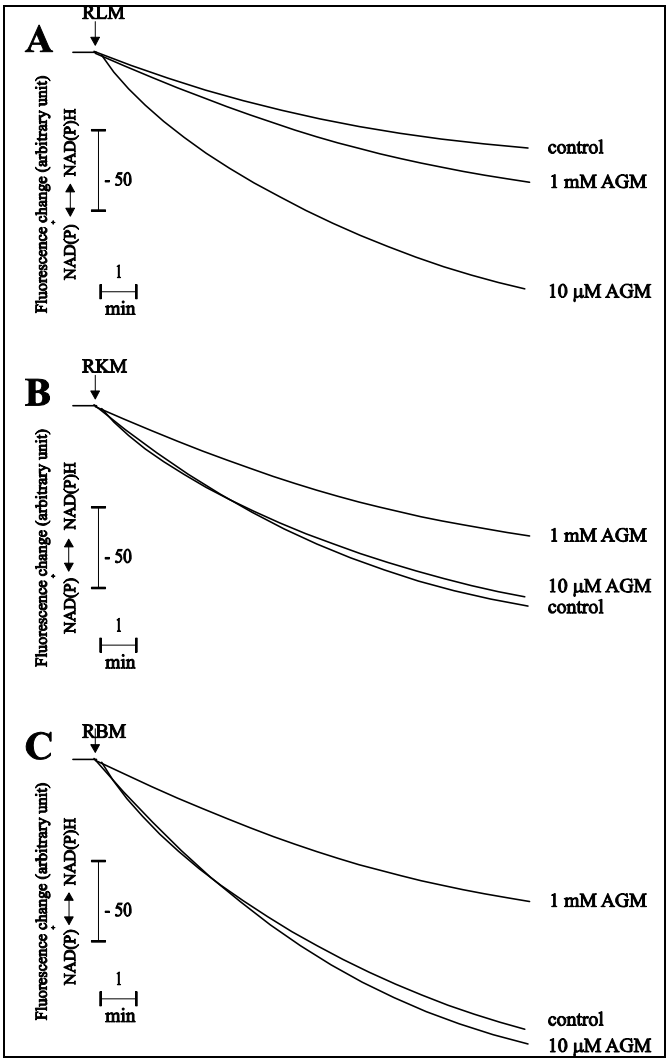


Fig. 23. Effect of agmatine on redox state of pyridine nucleotides in presence of Ca^{2+} in RLM (A), RKM (B), and RBM (C).
Mitochondria were incubated in standard medium, as described in Materials and Methods section, supplemented with 30 μM Ca^{2+}. Agmatine (AGM) concentrations are indicated at the side of traces. Values are the means ±SD of five experiments.

In RLM, 10 μM agmatine is also able to induce strong oxidation of pyridine nucleotides, whereas 1 mM is still ineffective (fig. 23 A). The pyridine nucleotides oxidation induced by Ca^{2+} in RKM and RBM (panels B and C) is protected by 1 mM agmatine, instead the lower concentrations are ineffective.

Then, agmatine at low concentrations (10, 100 μM) acts as a pro-oxidant agents in RLM, increasing MPT induction by Ca^{2+}; on the contrary, at the higher concentration (1 mM) agmatine behaves as a free radical scavenger, an effect exhibited also by polyamines [Sava et al., 2006]. Instead, agmatine protects RKM and RBM from MPT, demonstrating a capacity to act as a free radical scavenger, similar to the effect of higher concentration (1 mM) in RLM.

Discussion

In some cellular types, agmatine provokes polyamine depletion and suppression of cellular growth, in some cases also with the induction of apoptosis. Apoptosis induced by agmatine in hepatocytes is triggered by the release of cytochrome c from mitochondria and subsequent activation of caspase cascade [Gardini et al., 2001]. These effects are related to MPT induction in isolated RLM by low concentrations of agmatine (10-100 μM). Indeed, agmatine, at low concentrations, in the presence of Ca^{2+}, induces matrix swelling (fig. 20 A), an event related with the release of cytochrome c. Agmatine, in the absence of Ca^{2+}, provokes oxidative stress, evidenced by the generation of H_2O_2 (fig. 17 A), resulting in an increased oxygen uptake (fig. 16 A), and oxidation of thiol groups (fig. 18 A). All these effects are enhanced when Ca^{2+} is added to induce MPT. It should be noted that increased oxygen consumption by agmatine is associated with the regulation of urea synthesis, as demonstrated in perfused liver [Nissim et al., 2006]. This oxidation, however, does not damage the mitochondrial membrane, as $\Delta\Psi$ remains at normal levels (fig. 15 A). The involvement of an oxidative stress caused by agmatine is demonstrated by the protection of NEM and BHT, two well-known anti-oxidants, on thiol oxidation (fig. 18 A).

There are two possible explanation about agmatine action as oxidative agents. First, the induction of MPT by agmatine, at low concentrations, could be due to the presence of an amine oxidase in mitochondrial matrix which oxidizes agmatine to form H_2O_2 and, most probably, other ROS. The observation that the inhibitor of MAO activity, clorgyline, is ineffective against oxidation of thiols by agmatine, excludes the possible involvement of MAO (fig. 18 A). A recent study, moreover, hypothesize the presence of a new amine oxidase in mitochondria that can explain this effect [Cardillo et al., 2008]. Nevertheless, it is also possible the existence of another mechanism, already proposed for compounds with secondary amino-groups [Dalla Via et al., 2006]. These compounds form imino radicals by interacting with Fe^{3+} ions of the iron-sulfur centers present in respiratory complexes, so the radicals react with molecular oxygen generating ROS. A nitrogen present in the guanidine group of agmatine may behave in this way, by explaining the pro-oxidant effect of this amine [Battaglia et al., 2007].

A different behavior is exhibited by the high concentration of agmatine (1 mM) in RLM, which maintains the redox level of thiols and phosphorylation parameters as control (figs. 16 A, 18 A), and MPT is fully prevented (fig. 20 A). 1mM agmatine provokes only a slight increase in oxygen uptake, when compared with the lower

concentrations (fig. 16 A), most probably due to the reduced $\Delta\Psi$ drop caused by electrophoretic transport of the amine (fig. 15 A), and higher production of H_2O_2 (fig. 17 A). So, at higher concentrations, agmatine exhibits antioxidant properties, as also demonstrated for spermine [Sava et al., 2006]. In these conditions the amine can produce ROS but the amount of still unreacted molecules may act as a scavenger, by exhibiting self-protection against the ROS produced by itself. It is to note that the scavenging effect of spermine is direct against hydroxyl radical, leading to the conclusion that this ROS is responsible of the observed oxidative stress. The proposed mechanism is that agmatine, by reacting with hydroxyl radical, forms dihydroxyaminobutyl-guanidine which, by spontaneous dehydration and subsequent hydrolysis, forms guanidobutyric aldehyde. In conclusion, agmatine, at high concentration, can scavenge the ROS produced by Ca^{2+} and by itself. Moreover, the fact that agmatine acts as a scavenger of the hydroxyl radical explain also the dose-dependent production of H_2O_2 (fig. 17 A) which, most probably, reflects the parallel generation of hydroxyl radicals. These, however, are scavenged by unreacted agmatine – at a concentration of 1 mM – but remain integral and reactive at 10 and 100 μM.

In RKM and RBM, instead, agmatine, at all concentrations, does not provoke any alteration in bioenergetic parameters (fig. 16 B and C) or in oxidative status of thiol groups (fig. 18 B and C), and also does not causes the production of H_2O_2 (fig. 17 B and C). Moreover, in the presence of Ca^{2+}, agmatine exhibits a dose-dependent protection against MPT induction (fig. 20 B and C), and also against the subsequent effects: H_2O_2 production (fig. 21 B and C), and thiols (fig. 22 B and C) and pyridine nucleotides (fig. 23 B and C) oxidation. So, agmatine acts as free radical scavenger in kidney and brain mitochondria with the same mechanism proposed for liver.

The results obtained in RKM and RBM allow to clarify also the effect of low concentrations of agmatine in RLM. The explanation is that, in kidney and brain, due to lack of the above proposed amine oxidase, the oxidant effects by agmatine do not take place and the amine exhibits only the protective effect against the ROS produced by Ca^{2+}, and finally against MPT.

In considering the correlations between MPT induction and apoptosis, through the release of pro-apoptotic factors from intermembrane space following mitochondrial swelling, the pro-oxidant effects exhibited by agmatine at low concentrations in RLM explain the pro-apoptotic effect in hepatocytes cultures [Gardini et al., 2001]. The antiproliferative action exhibited by agmatine in different cell lines is ascribed, not only to a cytostatic effect due to polyamine depletion induced by the amine [Isome et al., 2007], but also to the activation of

caspase-3 and subsequent progression of apoptosis induced by agmatine [Wolf et al., 2007]. Indeed, the apoptosis progression is related to induction of MPT by the amine at low concentrations in isolated liver mitochondria.

On the contrary, some other studies reported a beneficial effects with agmatine administration in models of injury and inflammatory pathologies [Qiu and Zheng, 2006; Eto et al., 2006]. The capacity to reduce oxidative stress and protect mitochondrial functions exhibited by agmatine, in kidney and brain mitochondria and also at high concentration in liver, could explain these effects.

In conclusion, agmatine behaves as a regulator of cell energy content and triggers the apoptotic pathway through the regulation of MPT induction, above all by regulation of oxidative status of mitochondria.

3. Agmatine biosynthesis

Agmatine synthesis

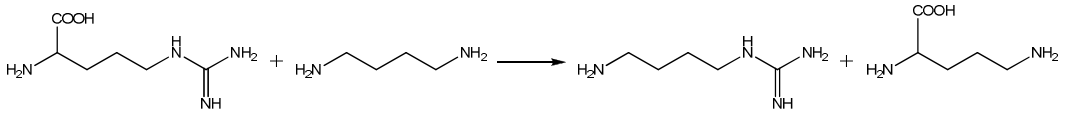
In plants, bacteria, and invertebrates, agmatine is formed by decarboxylation of L-arginine by the enzyme arginine decarboxylase (ADC; EC 4.1.1.19). In mammals, indeed, the agmatine synthesis is not yet well characterized and it is object of a debate between different authors.

Some authors described a mammalian ADC associated with mitochondrial membranes and capable to decarboxylate also ornithine but not inhibited by difluoromethylornithine (DFMO) [Regunathan and Reis, 2000]. Subsequently, they have characterized the sequence of this putative enzyme [Zhu et al., 2004] and the expression in brain region [Iyo et al., 2006]. Also other groups demonstrated an endogenous synthesis of agmatine in liver mitochondria by attributing it to ADC activity [Horyn et al., 2005].

On the contrary, other authors are in disagreement with these results as they were unable to demonstrate this activity in mammalian cells. Indeed, also the comparison of the genome databases, using non-mammalian ADC sequences, does not identify an ADC gene. In conclusion, a proposal is that agmatine can only be absorbed by the diet or produced by intestinal flora [Coleman et al., 2004].

It is known that agmatine may have important biological functions including the behaviour as a novel neurotransmitter, modulator of cellular proliferation and inflammation, regulator of renal and gastric function [Zhu et al., 2004] and also of polyamine metabolism. The existence of a biosynthetic pathway in mammalian cells would appear necessary for the amine to exhibit its functions.

In fact, I hypothesize the existence of a further enzyme for agmatine synthesis, a reaction very similar to that present in plants that involves a transaminidase enzyme [Srivenugopal and Adiga, 1980; Lee et al., 2000]. This reaction transfers an amidino group from arginine to putrescine to form agmatine:



Arginine Putrescine Agmatine Ornithine

In mammals, an enzyme similar to that of plants is the L-arginine:glycine amidinotransferase (EC 2.1.4.1), an enzyme involved in creatine pathway and mainly present in kidney with mitochondrial localization. This enzyme normally catalyze the reaction:

L-Arginine + Glycine ⟶ L-ornithine + Guanidine acetate

Moreover agmatine is present in kidney, in which acts as a functional regulator, by increasing the rate of proximal tubule filtration and glomerular reabsorption. The amine also stimulates Na^+/K^+ ATPase in kidney membrane preparations. Finally, the presence of imidazoline receptors localized to the basolateral aspect of proximal tubules was described [Lortie et al., 1996].

The contemporary presence of agmatine and amidinotransferase suggests the possibility that the enzyme is involved in agmatine synthesis. Then, the first investigation deal with the transport of the precursors, arginine and putrescine, in mitochondrial matrix. This transport is unique in kidney mitochondria.

The study is performed by considering the hypothesis for the presence of an amidinotransferase activity, both *in vivo*, in a transformed kidney proximal tubule cell line (MCT), and *in vitro*, in RKM lysate. The results report also a preliminary purification of the enzyme from RKM and kinetic studies on this activity.

Results

Arginine and putrescine transport in RKM

The presence of an amidinotransferase activity in RKM needs the presence of a specific transport of the precursors for agmatine synthesis for this reaction, arginine and putrescine.

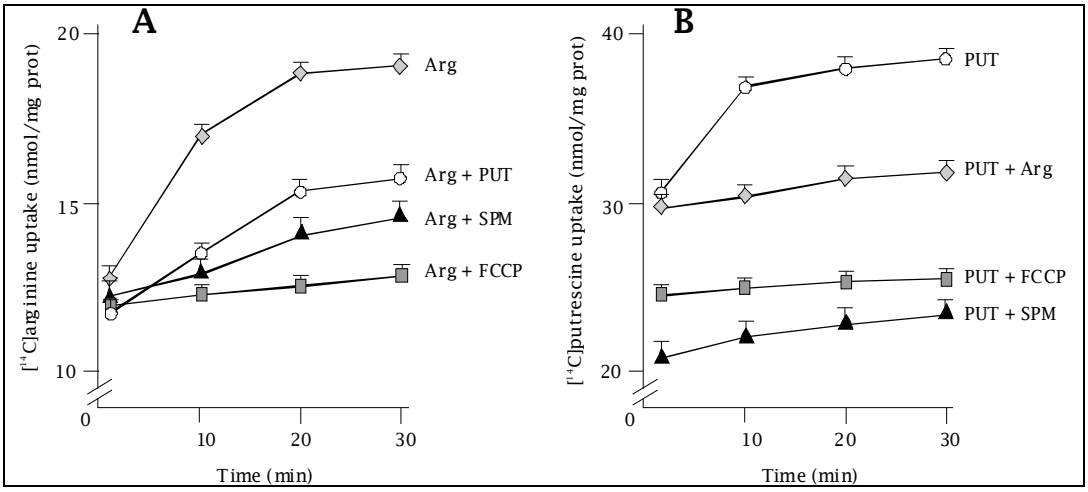


Fig. 24. Arginine (A) and putrescine (B) uptake by RKM.
RLM were incubated in standard medium, as described in Materials and Methods section, with 2 mM [^{14}C]arginine (50μCi/mmol) (A) and 2 mM [^{14}C]putrescine (50μCi/mmol) (B). When present in the medium: 0.1 μg/μl FCCP. Values are the means ±SD of three experiments.

The transport of arginine and putrescine is electrophoretic and dependent on mitochondrial membrane potential (see FCCP effect on fig. 24). Arginine and putrescine probably share the same transporter as each inhibits the transport of the other one. The transport of arginine is characteristic of kidney mitochondria, in fact in liver or heart mitochondria arginine transport almost negligible (unpublished results).

Agmatine synthesis in proximal tubule cell line

MCT cultures were starved O.N. and then cultured in 10% fBS for 24 h with 25 μM methylglyoxal bis(guanylhydrazone) (MGBG), an inhibitor of SAMDC and DAO, or with 5 mM DFMO, an ODC inhibitor. The addition of MGBG induces an increase in the endocellular putrescine content, while adding DFMO putrescine decreases. Subsequently, [^{14}C-guanide]arginine (0,60 mCi/mmol) was added to culture medium and, after 24 h, the cells were collected and treated for HPLC analysis.

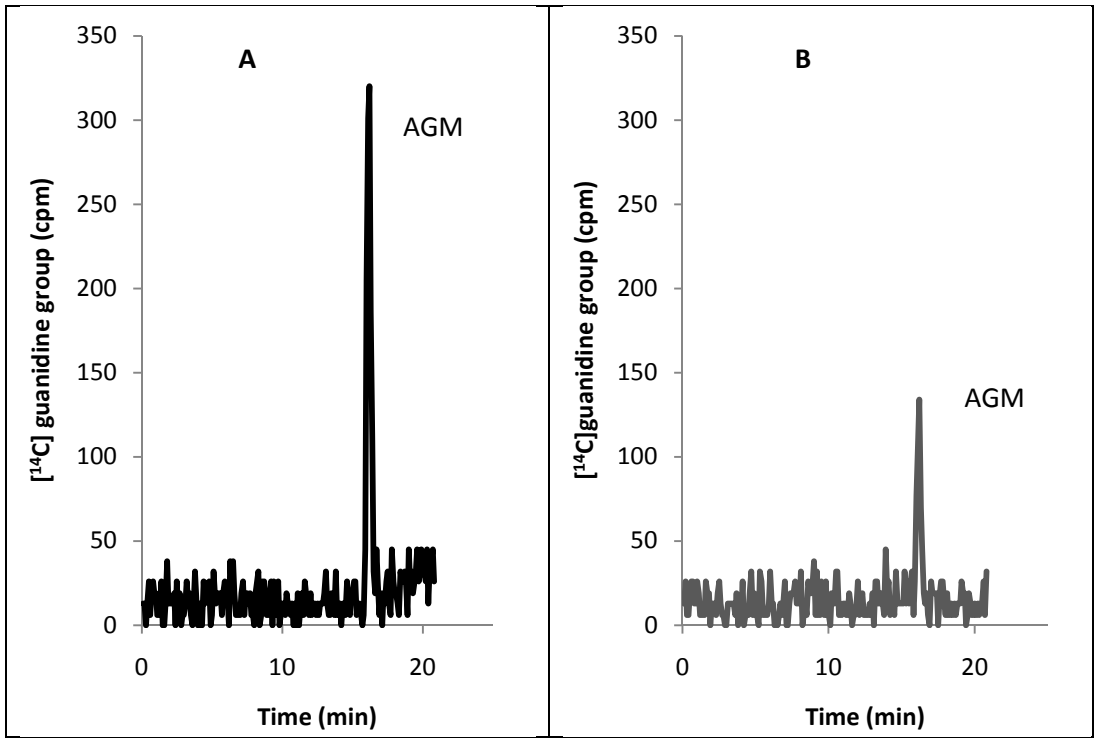


Fig. 25. Agmatine synthesis in MCT.
HPLC analysis of radiolabeled agmatine. Cells are incubated with [^{14}C-guanide]arginine (0,60 mCi/mmol). MCT treated with 25 μM MGBG (A) or 5 mM DFMO (B) for 24 h before adding radiolabeled arginine, then incubated for other 24 h. The sample preparation with buthanol extraction permit to identify a unique peak corresponding to agmatine (retention time 16.20 min).

[^{14}C]agmatine is synthesized in MCT when [^{14}C-guanide]arginine is added to culture medium. The results in fig. 25 evidence an increasing in agmatine content, in cells incubated with MGBG (panel A), if compared with those with DFMO (panel B). This confirms that agmatine synthesis can be related to the putrescine content in cells and suggests the presence of a biosynthetic reaction alternative to ADC.

Agmatine synthesis in RKM

RKM lysate was incubated in the presence of [^{14}C-guanide]arginine and cold putrescine. After 1 h of incubation the reaction was stopped and the samples were prepared for HPLC analysis.

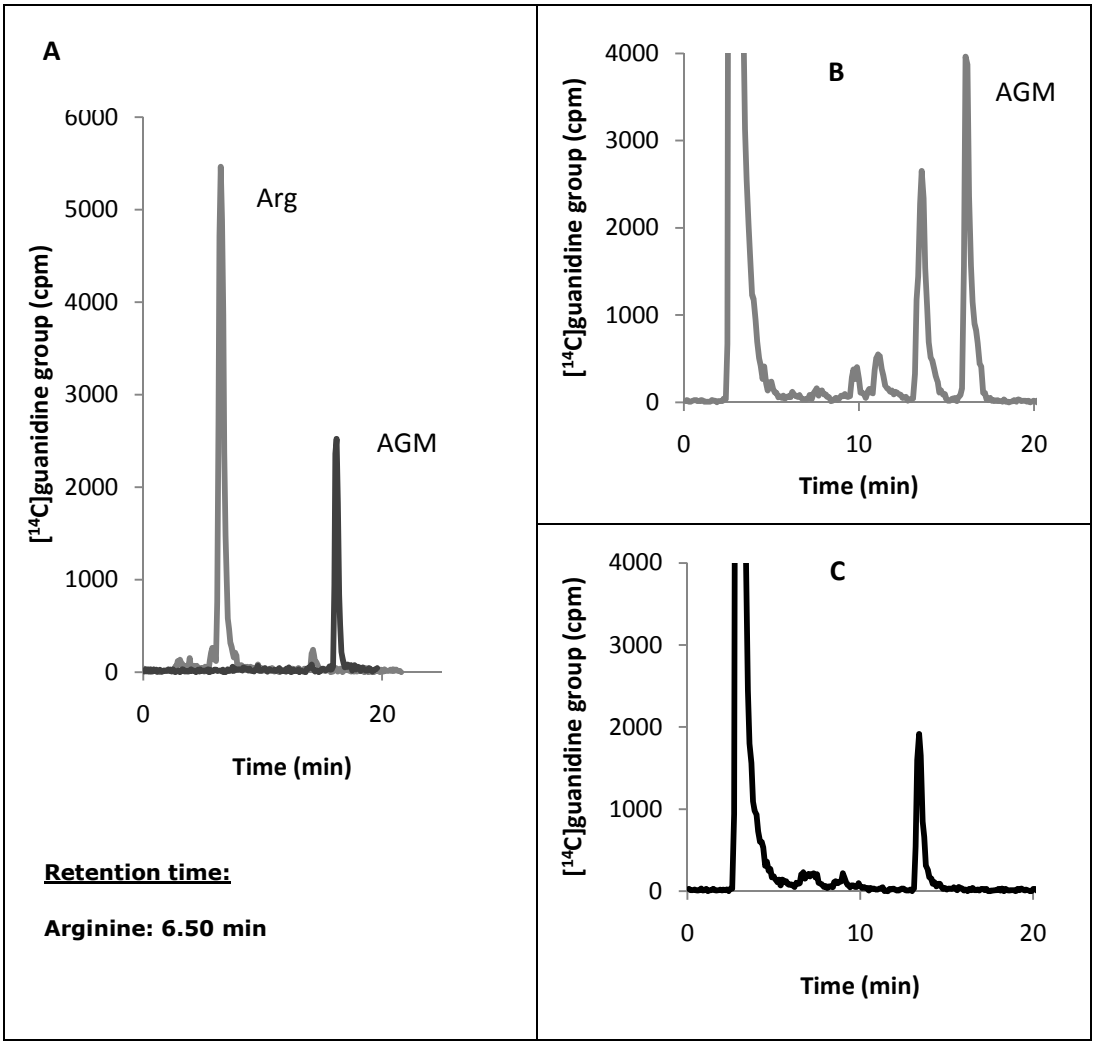


Fig. 26. Agmatine synthesis in RKM.
HPLC analysis of radiolabelled molecules. A: standards for [^{14}C]arginine and [^{14}C]agmatine. RKM lysate are incubated for 30 min in presence of 100 mM radiolabeled arginine (0,60 mCi/mmol) with (panel B) or without (panel C) 100 mM cold putrescine.

The results in panel B of fig. 26 demonstrate the presence of agmatine synthesis in RKM lysate. Indeed, a peak with the same retention time of agmatine standard (compare with panel A) is present when the lysates are incubated with [^{14}C-guanide]arginine and putrescine. In the absence of putrescine (panel C), agmatine is not formed. This indicates that also agmatine is synthesized by an amidinotransferase reaction and not by ADC.

Purification of amidino transferase

The amidinotransferase activity is increased by purification of RKM lysate using a FPLC method with a DEAE ionic exchange column, as described in Material and Methods section.

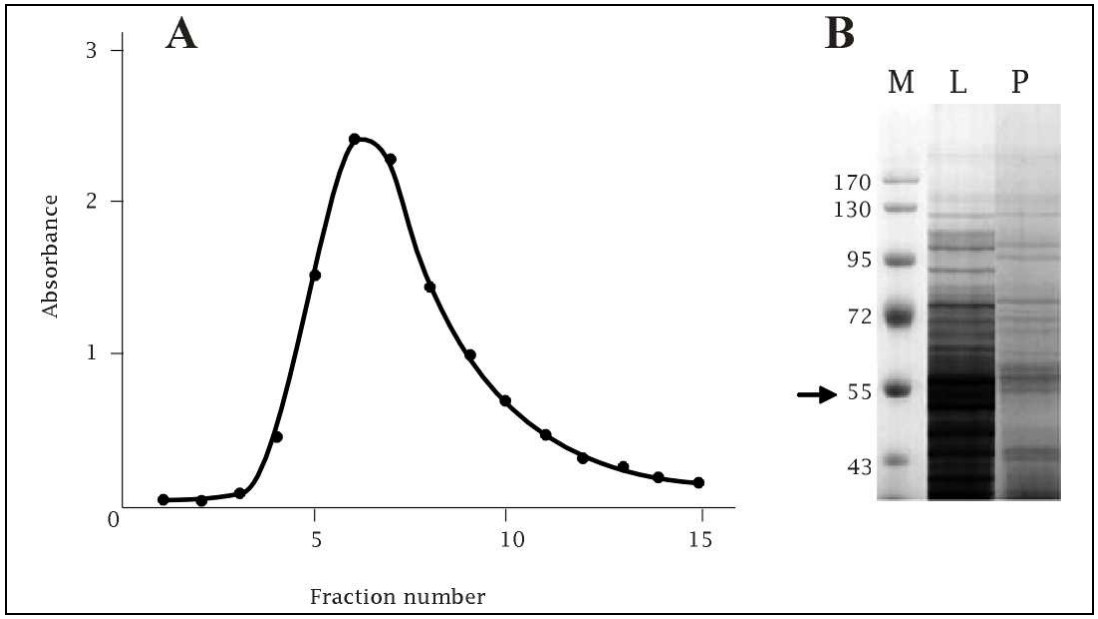


Fig. 27. Eluition profile of post column purificate.
A: Protein content in fractions obtained after DEAE-52 Cellulose column as described in Materials and methods section. B: Comassie blue coloration of SDS-PAGE gel. M=molecular weight markers; L=RKM lysate; P=fraction post column.

The elution profile in fig. 27 (panel A) demonstrates an enrichment in protein content in the fractions 4-9 which are also tested in SDS-PAGE to compare them with the original RKM lysate. The presence of a minor quantity of bands, and a more evident band corresponding to amidinotransferase (about 55 kDa), in the gel, is in agreement with an improved purification (panel B).

The fractions enriched in proteins (fractions 4-9) are used to measure amidinotrasferase activity as performed in RKM lysate.

Fraction	*Total protein* (mg)	*Total activity* (nmol/min)	*Specific activity* (nmol/min/ mg prot)	*Purification factor*	*Yield %*
RKM lysate	488,5	1754	3,59	/	/
Post DEAE	25,75	1200	46,6	12,98	68%

Table 1. Purification data.

As reported in table 1, the specific activity of amidino group transfer is notably increased in the post column fraction.

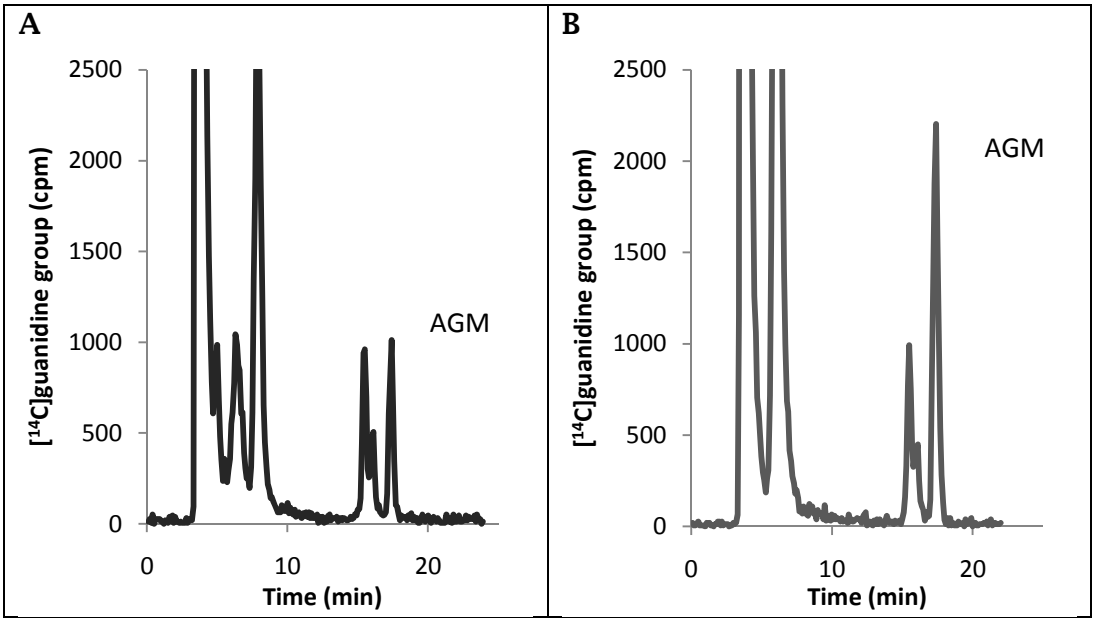


Fig. 28. Comparison between amidino transferase activity in RKM lysate (A) and post DEAE column purificate (B).
The incubation is performed as in fig. 26.

The comparison in fig. 28 between the two fractions, demonstrates an increase in the specific activity in the purified post DEAE column fraction (panel B), incubated with 100 mM putrescine in the same conditions as the RKM lysate (panel A). As observable in the figure, the peak corresponding to agmatine (retention time 16.20 min) is clearly higher in the purified fraction.

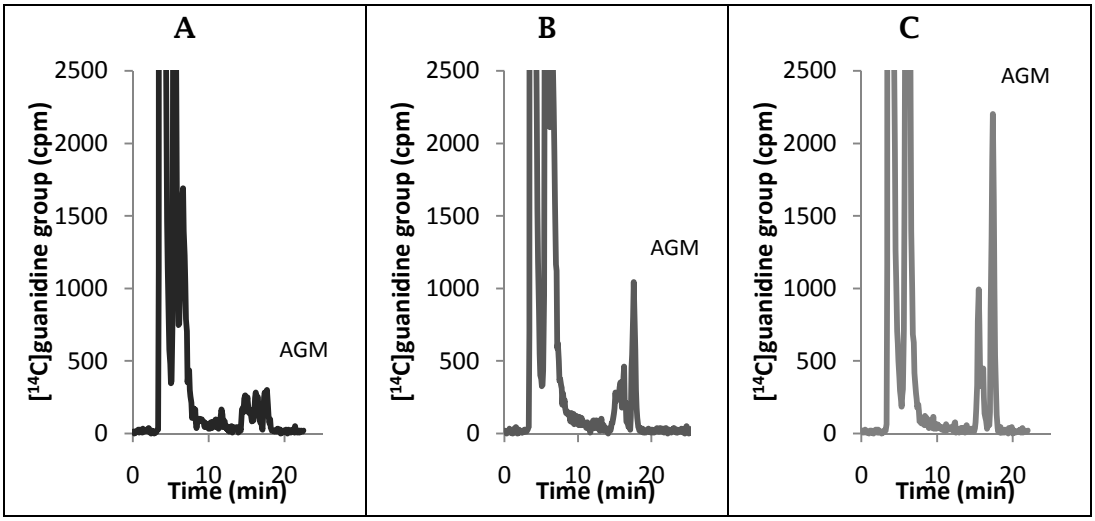


Fig. 29. Amidinotrasferase activity in the purificate with different putrescine concentrations.
The incubation is performed as in fig. 26, except to the concentrations of putrescine: 10 mM (A), 25 mM (B) and 100 mM (C).

The results in fig. 29 demonstrate the correlation between putrescine concentration and agmatine production by amidinotransferase reaction. The increase in the putrescine concentrations (from A to C), is accompanied by an increase of the peak corresponding to agmatine indicating a dose dependence of the reaction.

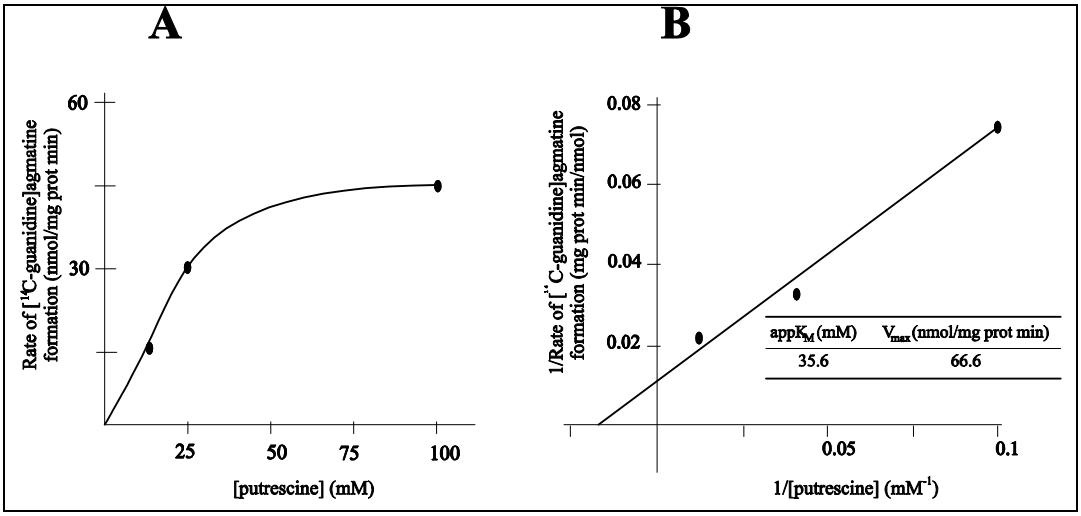


Fig. 30. Saturation kinetic (A) and double reciprocal plot (B) of amidinotransferase activity in the purificate.
The incubation is performed as in fig. 29. The activity of amidinotransferase reaction is linear over the incubation period.

Amidinotransferase activity exhibits a saturation kinetic and the calculated parameters from initial rates give appK_M of 35.6 mM and Vmax of 66.6 nmol/min · mg protein (fig. 30). The high appK_M value

may be due to the presence of arginase activity, normally bound to the outer mitochondrial membrane, thereby diminishing the availability of arginine for the reaction. Moreover, it is possible that the incubation conditions are not yet optimal or, I hypothesize the presence of an allosteric activator not even identified.

To obtain a higher activity, amidinotransferase has been undergone a second step of purification, using an affinity column able to bind putrescine. This step gives a very high level of purification, as the SDS gel exhibits a very evident band corresponding to amidinotransferase (about 55 kD). The specific activity of the amidinotransferase reaction is not increased, most probably due to a not optimal elution conditions. So, the isoform with amidinotransferase activity could be lost or the eluted enzyme is not stable in the final conditions.

Discussion

The endogenous synthesis of agmatine in mammalian cells is an unsolved question while in bacteria and plants a biosynthetic reaction, catalyzed by ADC, which decarboxylates arginine to form agmatine, is present.

Some authors proposed the presence of agmatine in mammals, suggesting a localization in mitochondrial inner membrane [Regunathan and Reis, 2000]. The group of Pegg, instead, was unable to confirm these results [Coleman et al., 2004], by claiming that the use of [^{14}C]arginine and the measurement of radiolabelled CO_2 produced by the reaction is not a good parameter for detecting ADC activity. Indeed, the most part of CO_2 is produced by other reactions. The lack of a putative ADC gene in mammalian genome should be a further confirmation that agmatine cannot be synthesized by ADC into mammalian cells.

However, another group reports the existence of agmatine synthesis in rat liver [Horyn et al., 2005], but the method used for the determination has been criticized. In any case the question remains open.

In plants there is an alternative enzyme for agmatine biosynthesis, due to the presence of a transaminidase [Srivenugopal and Adiga, 1980; Lee et al., 2000], which catalyzes the transfer of amidine group from arginine to different substrates, e.g. putrescine, with consequent formation of agmatine and ornithine. So, the production of labeled [^{15}N-guanide]agmatine, in the above mentioned study [Horyn et al., 2005], permits to hypothesize that this enzyme could be present also in mammalian cells. The enzyme more similar to plant transaminidase in mammals is the L-arginine-glycine:amidinotransferase (EC 2.1.4.1), involved in creatine pathway and expressed mainly in kidney with mitochondrial localization.

Agmatine has many biological functions and its distribution in tissue and organs is very differentiated. The lack of a clear demonstration of a biosyntethic pathway directly in mammals force to hypothesize that the amine is only absorbed by the diet or produced by intestinal flora, but is still hard to consider a differentiated uptake in tissues in these cases. Mammalian cells contain, on the contrary, the catabolic enzyme of agmatine: agmatinase, located in mitochondrial membranes. Thus, I hypothesize that all these characteristics are incompatible with the lacking of a biosynthesic pathway, while I propose the existence of an amidinotransferase activity to form agmatine also in kidney.

The preliminary results reported in this work demonstrate the presence of this amidinotransferase activity for the agmatine synthesis in RKM and MCT.

Furthermore the results here reported show that both putrescine and arginine are transported into the matrix of RKM by energy dependent mechanism (fig. 24). If putrescine transport in RKM cannot be considered a novelty as it has also been detected in mitochondria of other organs [Toninello et al., 1992], arginine transport is characteristic of RKM. Indeed, in other organs, the uptake of this amino acid is very low.

To test the dependence of agmatine synthesis on concentration of putrescine, I performed an experiment using MCT cells incubated in conditions that increase (with MGBG) or decrease (with DFMO) putrescine content. The increase of peak corresponding to radiolabeled agmatine (fig. 25) confirms that it is possible that agmatine is synthesized in this type of cells and also that this synthesis depends on putrescine concentration.

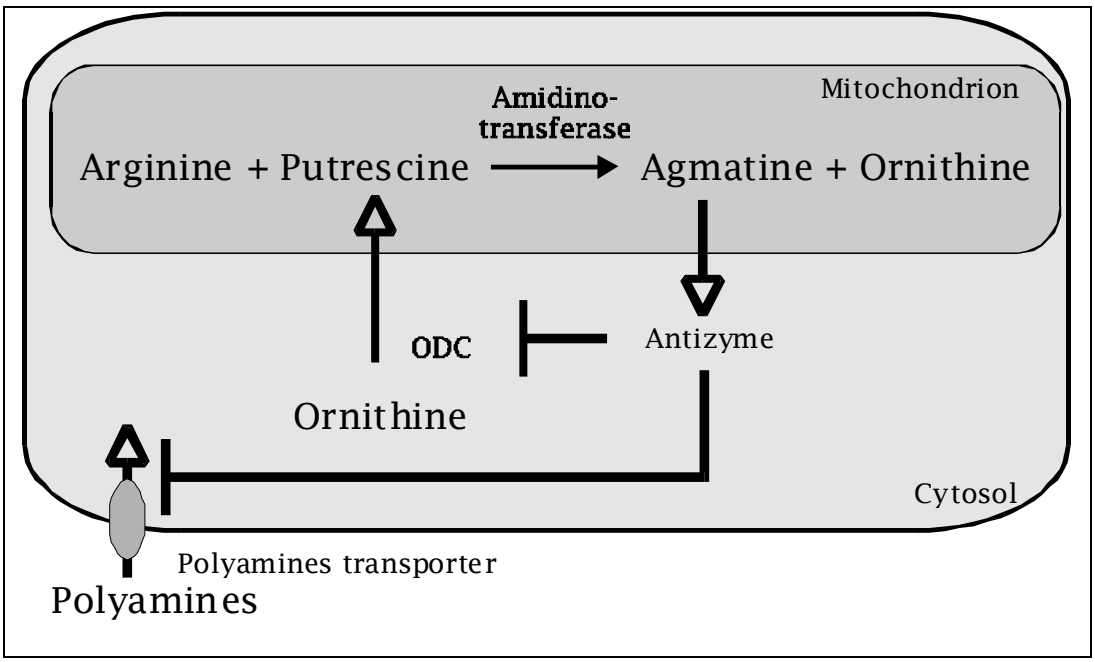
These first results are in agreement with the possibility that an amidinotransferase reaction is present to synthesize agmatine. The results in fig. 26 demonstrate that a RKM lysate incubated with [^{14}C-guanide]arginine and putrescine is able to produce a peak corresponding to agmatine. The lacking of this peak when the lysate is incubated without putrescine confirm that the amine is not produced by the activity of ADC but by an amidinotransferase reaction.

At this point I have performed a first preliminary purification of the enzyme according to methods already present in literature [Conconi and Grazi, 1965]. The results reported in figs. 27-28 and table 1, confirm the successful of the purification and the presence of a specific activity for amidinotransferase to synthesize agmatine. Moreover the reaction is dose-dependent with the putrescine concentration (fig. 28), and the kinetic analysis of the reaction gives a appK_M of 35.6 mM and V_{max} of 66.6 nmol/min ▪ mg protein (fig. 29). The calculation of these parameters has to be considered preliminary since the presence of arginase activity could be the cause of the very high appK_M. Moreover the incubation conditions couldn't be optimal and the use of radiolabeled molecules increases the difficulty to obtain these data. Now, I am performing other analysis to optimize the reaction conditions and to obtain more defined kinetic parameters.

One other step of purification is also in progress using an affinity column, but the elution conditions have to be improved, since after the second step the specific activity for amidinotransferase is not

increased. Probably, the isoform that synthesize agmatine is lost or the final conditions are not good for the enzyme reaction.

The preliminary results reported here confirm the presence of a reaction for agmatine synthesis in RKM independent to ADC. The existence of a biosynthetic pathway for agmatine in kidney could signify that the amine is not only a polyamine precursor, but could regulate the endocellular polyamine content by the subsequent feed-back mechanism:



In this model agmatine synthesis happens when in cells the putrescine concentration is very high. So, with the induction of ODC-antizyme, agmatine blocks the further production of putrescine by ODC and also the uptake of polyamines from extracellular space.

This hypothesis is in agreement with other studies demonstrating that exogenous agmatine in cultured cells is converted in very low amount to putrescine and cannot behave as an important polyamine precursor [Gardini et al., 2001]. Moreover, the agmatine administration in cultured cells determine the decrease in polyamine content and consequent block of the proliferation or apoptosis induction in proliferating and non-proliferating cells, respectively [Gardini et al., 2003; Isome et al., 2007].

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Supplement

Salvi M, Battaglia V, Mancon M, Colombatto S, Cravanzola C, Calheiros R, Marques MPM, Grillo MA, Toninello A, 2006. Agmatine is transported into liver mitochondria by a specific electrophoretic mechanism, Biochem J, 396:337-345.

Agmatine, a divalent diamine with two positive charges at physiological pH, is transported into the matrix of liver mitochondria by an energy-dependent mechanism the driving force of which is DeltaPsi (electrical membrane potential). Although this process showed strict electrophoretic behaviour, qualitatively similar to that of polyamines, agmatine is most probably transported by a specific uniporter. Shared transport with polyamines by means of their transporter is excluded, as divalent putrescine and cadaverine are ineffective in inhibiting agmatine uptake. Indeed, the use of the electroneutral transporter of basic amino acids can also be discarded as ornithine, arginine and lysine are completely ineffective at inducing the inhibition of agmatine uptake. The involvement of the monoamine transporter or the existence of a leak pathway are also unlikely. Flux-voltage analysis and the determination of activation enthalpy, which is dependent upon the valence of agmatine, are consistent with the hypothesis that the mitochondrial agmatine transporter is a channel or a single-binding centre-gated pore. The transport of agmatine was non-competitively inhibited by propargylamines, in particular clorgilyne, that are known to be inhibitors of MAO (monoamine oxidase). However, agmatine is normally transported in mitoplasts, thus excluding the involvement of MAO in this process. The I2 imidazoline receptor, which binds agmatine to the mitochondrial membrane, can also be excluded as a possible transporter since its inhibitor, idazoxan, was ineffective at inducing the inhibition of agmatine uptake. Scatchard analysis of membrane binding revealed two types of binding site, S1 and S2, both with mono-co-ordination, and exhibiting high-capacity and low-affinity binding for agmatine compared with polyamines.Agmatine transport in liver mitochondria may be of physiological importance as an indirect regulatory system of cytochrome c oxidase activity and as an inducer mechanism of mitochondrial-mediated apoptosis.

Toninello A, Battaglia V, Salvi M, Calheiros R, Marques MPM, 2006. Structural characterization of agmatine at physiological conditions, Struc Chem, 17:163-175.

The present work aims at determining the structure–activity relationships (SAR's) which rule the biological function of agmatine (4-(aminobutyl)guanidinium, AGM), a biogenic amine produced by decarboxylation of arginine. Its structural preferences, both as an isolated molecule and in aqueous solution (namely at physiological conditions) were ascertained, by vibrational (Raman) spectroscopy coupled to theoretical (density functional) calculations. An evaluation of mitochondrial functions (membrane potential ($\Delta\Psi$), mitochondrial swelling, and cytochrome c release) in rat liver mitochondria (RLM) was also carried out. The results thus obtained, coupled to the conformational analysis performed for the distinct polyamine protonation states, allowed to individualize the agmatine structures which interact with the mitochondrial site responsible for its transport and for the protection against mitochondrial permeability transition (MPT) induction, as well as to gain information on the specific mechanisms involved.

Battaglia V, Rossi CA, Colombatto S, Grillo MA, Toninello A, 2007. Different behavior of agmatine in liver mitochondria: inducer of oxidative stress or scavenger of reactive oxygen species?, Biochim Biophys Acta, 1768:1147-1153.

Agmatine, at concentrations of 10 microM or 100 microM, is able to induce oxidative stress in rat liver mitochondria (RLM), as evidenced by increased oxygen uptake, H(2)O(2) generation, and oxidation of sulfhydryl groups and glutathione. One proposal for the production of H(2)O(2) and, most probably, other reactive oxygen species (ROS), is that they are the reaction products of agmatine oxidation by an unknown mitochondrial amine oxidase. Alternatively, by interacting with an iron-sulfur center of the respiratory chain, agmatine can produce an imino radical and subsequently the superoxide anion and other ROS. The observed oxidative stress causes a drop in ATP synthesis and amplification of the mitochondrial permeability transition (MPT) induced by Ca(2+). Instead, 1 mM agmatine generates larger amounts of H(2)O(2) than the lower concentrations, but does not affect RLM respiration or redox levels of thiols and glutathione. Indeed, it maintains the normal level of ATP synthesis and prevents Ca(2+)-induced MPT in the presence of phosphate. The self-scavenging effect against ROS production by agmatine at higher concentrations is also proposed.

Grillo MA, Battaglia V, Colombatto S, Rossi CA, Simonyan AR, Salvi M, Khomutov AR, Toninello A, 2007. Inhibition of agmatine transporter in liver mitochondria by new charge-deficient agmatine analogues, Biochem Soc Trans, 35:401-404.

The charge of the agmatine analogues AO-Agm [N-(3-aminooxypropyl)guanidine], GAPA [N-(3-aminopropoxy)guanidine] and NGPG [N-(3-guanidinopropoxy)guanidine] is deficient as compared with that of agmatine and they are thus able to inhibit agmatine transport in liver mitochondria. The presence of the guanidine group is essential for an optimal effect, since AO-Agm and NGPG display competitive inhibition, whereas that of GAPA is non-competitive. NGPG is the most effective inhibitor (K(i)=0.86 mM). The sequence in the inhibitory efficacy is not directly dependent on the degree of protonation of the molecules; in fact NGPG has almost the same charge as GAPA. When the importance of the guanidine group for agmatine uptake is taken into account, this observation suggests that the agmatine transporter is a single-binding, centre-gated pore rather than a channel.

Pezzato E, Battaglia V, Brunati AM, Agostinelli E, Toninello A, 2008. Ca(2+)-independent effects of spermine on pyruvate dehydrogenase complex activity in energized rat liver mitochondria incubated in the absence of exogenous Ca(2+) and Mg (2+), Amino Acids, e-pub.

In the absence of exogenous Ca(2+) and Mg(2+) and in the presence of EGTA, which favours the release of endogenous Ca(2+), the polyamine spermine is able to stimulate the activity of pyruvate dehydrogenase complex (PDC) of energized rat liver mitochondria (RLM). This stimulation exhibits a gradual concentration-dependent trend, which is maximum, about 140%, at 0.5 mM concentration, after 30 min of incubation. At concentrations higher than 0.5 mM, spermine still stimulates PDC, when compared with the control, but shows a slight dose-dependent decrease. Changes in PDC stimulation are very close to the phosphorylation level of the E(1alpha) subunit of PDC, which regulates the activity of the complex, but it is also the target of spermine. In other words, progressive dephosphorylation gradually enhances the stimulation of RLM and progressive phosphorylation slightly decreases it. These results provide the first evidence that, when transported in RLM, spermine can interact in various ways with PDC, showing dose-dependent behaviour. The interaction most probably takes place directly on a specific site for spermine on one of the regulatory enzymes of PDC, i.e. pyruvate dehydrogenase phosphatase (PDP). The interaction of spermine with PDC may also involve activation of another regulatory enzyme, pyruvate dehydrogenase kinase (PDK), resulting in an increase in E(1alpha) phosphorylation and consequently reduced stimulation of PDC at high polyamine concentrations. The different effects of spermine in RLM are discussed, considering the different activities of PDP and PDK isoenzymes. It is suggested that the polyamine at low concentrations stimulates the isoenzyme PDP(2) and at high concentrations it stimulates PDK(2).

Sava IG, Battaglia V, Rossi CA, Salvi M, Toninello A, 2006. Free radical scavenging action of the natural polyamine spermine in rat liver mitochondria, Free Rad Biol Med, 41:1272-1281.

The isoflavonoid genistein, the cyclic triterpene glycyrrhetinic acid, and salicylate induce mitochondrial swelling and loss of membrane potential (Delta Psi) in rat liver mitochondria (RLM). These effects are Ca(2+)-dependent and are prevented by cyclosporin A and bongkrekik acid, classic inhibitors of mitochondrial permeability transition (MPT). This membrane permeabilization is also inhibited by N-ethylmaleimide, butylhydroxytoluene, and mannitol. The above-mentioned pro-oxidants also induce an increase in O(2) consumption and H(2)O(2) generation and the oxidation of sulfhydryl groups, glutathione, and pyridine nucleotides. All these observations are indicative of the induction of MPT mediated by oxidative stress. At concentrations similar to those present in the cell, spermine can prevent swelling and Delta Psi collapse, that is, MPT induction. Spermine, by acting as a free radical scavenger, in the absence of Ca(2+) inhibits H(2)O(2) production and maintains glutathione and sulfhydryl groups at normal reduced level, so that the critical thiols responsible for pore opening are also consequently prevented from being oxidized. Spermine also protects RLM under conditions of accentuated thiol and glutathione oxidation, lipid peroxidation, and protein oxidation, suggesting that its action takes place by scavenging the hydroxyl radical.