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Different behaviour of liver and brain mitochondria in Permeability Transition: role of biogenic monoamines

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Abstract

Biologically active amines are a class of compounds synthesized by normal metabolic processes in living organisms. They are classified as biogenic amines (serotonin, agmatine, tyramine, histamine, dopamine, phenylethylamine, tryptamine and catecholamines), or polyamines (putrescine, spermidine and spermine). Biogenic amines can act as neurotransmitters and display various other physiological functions throughout the organism. The degradation of these molecules is catalyzed by monoamine oxidases (MAOs) A and B, isoenzymes localized on outer mitochondrial membrane, which induce an oxidative deamination. This reaction leads to the production of hydrogen peroxide and aldehydes, which are then oxidized into acids or converted into alcohols or glycols. In particular hydrogen peroxide can trigger the formation of other reactive oxygen species (ROS) and induce mitochondrial damages and apoptosis. Considering that biogenic amines can undergo these catabolic reactions, the possible effects of them, or of their products, on different types of mitochondria, were studied.

The aim of this work was to study the action of monoamines as regulator of mitochondrial functions in isolated rat mitochondria from different organs: liver, brain, heart, and kidney.

The first part of the work focused on the action of these amines on mitochondrial permeability transition induction. They induce a collapse of $\Delta\Psi$ and a strong amplification of swelling in rat isolated mitochondria of liver (RLM), heart (RHM) and kidney (RKM). Furthermore they oxidize thiol groups and pyridine nucleotides. These observations support the hypothesis that monoamines are amplifiers of mitochondrial permeability transition (MPT), inducing an oxidative stress, through the generation of H₂O₂, which is most probably the agent responsible of MPT occurrence. Instead, in isolated rat brain mitochondria (RBM), the amines do not amplify the swelling and do not alter the partial drop of $\Delta\Psi$ induced by Ca²⁺, even if they oxidize thiol groups and pyridine nucleotides. These results led us to hypothesize the existence in RBM of a mechanism of MPT pore opening different from that present in the other mitochondria.

In the second part of the study it is reported the serotonin uptake by mitochondria with characterization of the transport system. Experimental evidences suggesting that aldehyde is the possible accumulated species are reported.

Finally, in the third part of the work, in order to better define the process that triggers MPT in RBM we have investigated the role of signaling pathways, in particular the possible involvement of P-Tyr-phosphorilated proteins since it has been reported that this type of mitochondria contains Tyr-kinases of the Src-family. We found, on the one hand, that a variety of tyrosine-kinase inhibitors do not affect the process while the "Inhibitor Tyr-phosphatases Cocktail 2", and the known phosphatase inhibitor sodium-pervanadate reduce the occurrence of MPT in parallel with an increase of the P-Tyr level of some proteins, in particular of proteins of apparent M.W. of 160, 72 and 35 kDa. Experiments are in progress to define, first of all, the identity of the P-Tyr-Protein involved in this process and then the characteristics and physiological significance of this phenomenon.

In conclusion the obtained results show an important role of monoamines in mitochondria that depends on the tissues and their specific physiological processes.

Furthermore two different mechanisms seem to be involved in MPT. In RLM the opening of permeability transition pore appears to require oxidation of thiol groups and the MPT amplification seems to depend on the oxidative stress induced by the reactive oxygen species produced by monoamine oxidation. In RBM the pore opening seems to involve two different mechanisms: in addition to the oxidative stress also the Tyr-phosphorylation of some proteins whose nature is under investigation.

Sommario

Le amine biologicamente attive sono una classe di composti sintetizzati negli organismi viventi da normali processi metabolici. Esse sono classificate come amine biogene: serotonina, agmatina, tiramina, istamina, dopamina, feniletilamina, triptamina e catecolamine, o poliamine : putrescina, spermidina e spermina. Le amine biogene possono agire come neurotrasmettitori e dimostrano altre funzioni fisiologiche in diversi organi. La degradazione di queste molecole è catalizzata dalle monoamino-ossidasi (MAO) A e B, isoenzimi localizzati sulla membrana esterna mitocondriale, che inducono una deaminazione ossidativa. Questa reazione porta alla produzione di perossido di idrogeno e delle corrispondenti aldeidi, che vengono poi ossidate ad acidi o ridotte ad alcoli o glicoli. In particolare, il perossido di idrogeno può innescare la formazione di altre specie reattive dell'ossigeno (ROS) e indurre danni mitocondriali e apoptosi. Considerando che le amine biogene possono subire queste reazioni cataboliche, i loro possibili effetti, o quelli dei loro prodotti di ossidazione, sono stati studiati sui diversi tipi di mitocondri.

Lo scopo di questo studio è stato quello di studiare l'azione delle monoamine come regolatrici delle funzioni mitocondriali nei mitocondri isolati da differenti organi di ratto: fegato, cervello, cuore e reni.

La prima parte del lavoro si concentra su l'azione di queste ammine sull'induzione di transizione di permeabilità mitocondriale. Esse producono un crollo del potenziale elettrico di membrana ($\Delta\Psi$) e una forte amplificazione dello swelling di mitocondri isolati di fegato (RLM), cuore (RHM) e reni (RKM) di ratto. Inoltre le amine ossidano i gruppi tiolici e i nucleotidi piridinici. Queste osservazioni supportano l'ipotesi che le monoamine siano amplificatrici della transizione di permeabilità mitocondriale (MPT), inducendo uno stress ossidativo, attraverso la generazione di H₂O₂. Quest'ultimo composto sembra essere, molto probabilmente, l'agente responsabile dell'induzione della MPT. Invece, nei mitocondri di cervello di ratto (RBM), le amine non amplificano lo swelling e non alterano il parziale calo di $\Delta\Psi$ indotti dal Ca²⁺, nonostante i gruppi tiolici e i piridin nucleotidi vengano ossidati come nei RLM. Questi risultati ci hanno portato ad ipotizzare l'esistenza nei RBM di un meccanismo di apertura del poro di transizione diverso da quello presente negli altri tipi mitocondriali.

Nella seconda parte dello studio è riportato il trasporto della serotonina nei mitocondri con la caratterizzazione del sistema di trasporto. Evidenze sperimentali suggeriscono che sia l'aldeide derivata dalle monoamine la possibile specie accumulata.

Infine, nella terza parte del lavoro, al fine di definire meglio il processo che innesca la MPT nei RBM, abbiamo studiato il ruolo delle vie di trasmissione del messaggio, in particolare il possibile coinvolgimento di proteine tirosin fosforilate, anche in base al fatto che è stato riportato che questo tipo di mitocondri contiene Tyr-chinasi della famiglia Src. Abbiamo trovato, da un lato, che una serie di inibitori delle tirosin-chinasi non influenzano la MPT, mentre l'"Inhibitor Tyr-phosphatases Cocktail 2", e il noto inibitore delle fosfatasi pervanadato riducono l'insorgenza di tale processo in parallelo con un aumento del livello P-Tyr di alcune proteine, in particolare, proteine di un apparente massa molecolare 160, 72 e 35 KDa. Esperimenti sono in corso per definire, prima di tutto, l'identità delle proteine fosforilate in tirosina coinvolte in questo processo e quindi le caratteristiche e il significato fisiologico di questo fenomeno.

In conclusione i risultati ottenuti mostrano un ruolo importante delle monoamine nei mitocondri che dipende dai tipi di tessuto e dai loro specifici processi fisiologici.

Inoltre sembrano essere coinvolti nel processo della MPT due diversi meccanismi. Nei RLM l'apertura del poro di transizione della permeabilità sembra richiedere l'ossidazione dei gruppi tiolici e l'amplificazione della MPT sembra dipendere dallo stress ossidativo indotto da specie reattive dell'ossigeno prodotte dall'ossidazione delle monoamine. Nei RBM l'apertura del poro sembra invece dipendere da due diversi meccanismi: oltre che dallo stress ossidativo anche dalla fosforilazione tirosinica di alcune proteine sulla cui natura si sta attualmente indagando.

Abbreviations

| AdNT | adenine nucleotide translocase |
|------------|---|
| AIF | apoptosis inducing factors |
| Amplex Red | 10-acetyl-3,7-dihydroxyphenoxazine |
| ВНТ | butyl-hydroxytoluene |
| ВКА | bongkrekic acid |
| BSA | bovine serum albumine |
| CsA | cyclosporin A |
| СурD | cyclophylin D |
| DA | dopamine |
| DTNB | 5,5'-dithio-bis-2-nitrobenzoic acid |
| FCCP | carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone |
| HRP | horseradish peroxidase |
| ΜΑΟ | monoamine oxidase |
| МРТ | mitochondrial permeability transition |
| NEM | N-ethylmaleimide |
| PAGE | polyacrilamide gel electrophoresis |
| PBS | phosphate buffered saline |
| Pi | phosphate |
| PP2 | 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine |
| РТР | permeability transition pore |
| RBM | rat brain mitochondria |
| RCI | respiratory control index |
| RHM | rat heart mitochondria |

| RKM | rat kidney mitochondria |
|--------------------|---|
| RLM | rat liver mitochondria |
| ROS | reactive oxygen species |
| SDS | sodium dodecyl sulphate |
| SER | serotonin |
| TYR | tyramine |
| TPP ⁺ | tetraphenylphosphonium |
| ΔΕ | electrode potential variation |
| ΔΨ | mitochondrial electric membrane potential |
| $\Delta {\mu_H}^+$ | electrochemical gradient |

Introduction

Monoamines

Biologically active amines are a class of compounds which are not only synthesized during normal metabolic processes in living organisms, but they are also taken up from dietary exogenous sources. Active amines have been classified as *biogenic*: agmatine, serotonin, tyramine histamine, tryptamine and catecholamines or *polyamines*: putrescine, spermidine, spermine by COST action 917 and 922 (Cooperation in Science and Technology, research programs, financed by the Commission of European Community DG/XIIB for studies on biologically active amines in food). These amines have various characteristics and physiological functions including an important role in cellular growth and differentiation, for this reason they are considered as biological regulators. The biogenic amines considered in this thesis are serotonin, tyramine and dopamine. They contain one amino group that is linked to an aromatic ring by a two-carbon chain (-CH2-CH2-). These monoamines are derived from aromatic amino acids tryptophan (serotonin) and tyrosine (dopamine and tyramine).

<u>Serotonin</u>

Serotonin (5-hydroxytryptamine, 5-HT, SER) is a monoamine neurotransmitter synthesized in neurons of the central nervous system (CNS) and in enterochromaffin cells of the gastrointestinal tract. It is involved in the regulation of various psychological and physiological states such as anger, mood, sexuality, appetite, sleep and body temperature (Jacobs and Azmitia, 1992). Serotonin plays important roles through several membrane-bound receptors present both in the central and peripheral nervous system, as well as in other tissues. It exerts its effects on blood vessels too. 5-HT directly contracts arteries and veins on smooth muscular cells by 5-HT_{2A} receptors. It is released at the synapses and its reuptake is mediated by the specific transporter SERT located in the presynaptic nerve terminals. Platelets express SERT which allow them to sequester serotonin from the environment; this transporter exhibits the same structural and functional properties of the serotoninergic nerve ending transporter (Affolter and Pletscher 1982; Da Prada et al. 1988; Rudnick and Clark

1993). Platelets constitute the major storage of 5-HT even if they are not able to synthesize it (Da Prada et al. 1988).

5-HT is synthesized from the amino acid L-tryptophan by a short metabolic pathway mediated by two enzymes: tryptophan hydroxylase (TPH) and amino acid decarboxylase (DDC). The TPH-mediated reaction is the rate-limiting step of the process (Fig. 1).



Fig. 1 Biosynthesis of serotonin.

Dopamine

Dopamine (3,4-dihydroxyphenethylamine; DA) is a biogenic amine of catecholamine family.

DA displays several functions through the body and it acts as a CNS neurotransmitter for neurons involved in regulating movement (nigrostriatal pathway) and motivated behavior (mesolimbic pathway) (Wise 2004; Robinson et al. 2006). DA is a central component of neuroendocrine axes (hypothalamus) and it serves as an intermediate in the synthesis of both epinephrine and norepinephrine, which belong to the same catecholamine family, in the peripheral and central nervous system (Hornykiewicz 1966). Several *in vitro* and *in vivo* studies demonstrated that DA is a toxic molecule that may contribute to neurodegenerative disorders such as ischemia-induced striatal damage and Parkinson's disease (Dukes et al. 2008; Hattoria et al. 2009). It is already known

that the metabolism of this neurotransmitter produces reactive oxygen species (hydroxyl radical, peroxide and superoxide) (ROS), but growing evidence suggests that DA itself can play a direct role in the neurodegenerative process (Graham et al. 1978). DA is synthesized in the cytoplasm first by the hydroxylation of the amino acid L-tyrosine to L-DOPA via the enzyme tyrosine 3-monooxygenase, also known as tyrosine hydroxylase, and then by the decarboxylation of L-DOPA by aromatic L-amino acid decarboxylase (which is referred to as dopa decarboxylase). In some neurons, DA is further processed into norepinephrine by dopamine beta-hydroxylase (Fig. 2). Subsequently, it is immediately packed into monoaminergic vesicles by the vesicular monoamine transporter (VMAT2) and then released into the synaptic cleft, where it can bind to dopamine receptors located on the postsynaptic membrane. Afterwards it can be taken up by the dopamine transporter (DAT) and returned back to the cytoplasm of the presynaptic neuron.

Tyramine

Tyramine (4-hydroxyphenethylamine, TYR) is a naturally occurring monoamine compound and trace amine derived from the amino acid tyrosine. Trace amines metabolically related to classical are structurally and monoamine neurotransmitters, such as dopamine, norepinephrine, and serotonin, they are endogenously present in trace concentrations. They are distributed heterogeneously throughout the mammalian brain and peripheral nervous tissues and exhibit high rates of metabolism. Although, they can be synthesized within parent monoamine neurotransmitter systems, there is evidence suggesting that some of them may comprise their own independent neurotransmitter systems Trace amines may play very significant roles in the coordination of biogenic monoamine-based synaptic physiology (Burchett and Hicks 2006). A family of G protein coupled receptors known as TAARs (trace amine associated receptors) has been characterized to be responsive to trace amines (Lindemann et al. 2005) and structurally related psychoactive drugs, such as amphetamine, MDMA, LSD, and DMT (Bunzow et al. 2001). The trace amines have been implicated in a vast array of human disorders of affect and cognition, such as depression(Davis and Boulton 1994) and schizophrenia (O'Reilly and Davis 1994). TYR acts as a catecholamine releasing agent. However, it is unable to cross the blood-brain-barrier, producing only non-psychoactive peripheral sympathomimetic effects. Biochemically, TYR is produced by the decarboxylation of tyrosine via the action of the enzyme tyrosine decarboxylase, it is then

degraded by tyramine beta-hydroxylase (into octopamine Fig. 2), which is subsequently packaged in synaptic vesicles with norepinephrine. Tyramine is physiologically metabolized by MAO-A.





The level of these compounds is controlled by MAOs that are flavoenzymes tightly associated with the outer mitochondria membrane, bound by a C-terminal transmembrane polypeptide segment (Mitoma and Ito 1992). Two isoforms exhist: MAO-A and MAO-B composed of 527 and 520 amino acids, respectively, and have a 70% amino acid identity (Bach et al. 1988). Under normal physiological conditions MAO-A preferentially oxidizes serotonin whereas dopamine and tyramine are substrates for both isoenzymes. Their distribution is very wide, it is well documented their presence in brain of adult organisms where they are important for the catabolism of neurotransmitters (Abell and Kwan 2001). High activities of MAO-A are also found in placenta and liver as well as kidney, adrenal gland, heart and lung. Activity of MAO-B is found to be lower

than MAO-A in all tissues except skeletal muscle, where both enzyme levels are similar (Billett 2004).

<u>Apoptosis</u>

Apoptosis (Greek: apo - from/off/without, ptosis - falling), describes the action of 'falling away from' similar to the leaves falling away from a tree. This term has been first used by Kerr, Wyllie and Currie in 1972 and was defined as an innately original type of cell death that is responsible from cell loss in living tissues (Kerr et al. 1972). Apoptosis is a mechanism of cellular death far distinct from necrosis, which is known to be the classical mechanism of cell death (Cotter et al. 1990). The significance of apoptosis in important physiological processes, such as normal cell turnover, developmental biology, and functions of the immune system, brought about to an increased interest on this topic. It shows distinct morphological characteristics, for instance, cell shrinkage is observed during its establishment as well as the chromatin aggregation and condensation around the nuclear membrane (Majno and Joris 1995). In apoptotic cells the membrane remains intact and shows irregular buds, which are known as membrane blebs, and small pieces known as apoptotic bodies are also observed. These bodies are surrounded by a membrane and contain variable amounts of nuclear material and other intracellular content. Apoptotic bodies are phagocytosed by macrophages or the neighboring cells, thus an inflammatory response does not occur (Majno and Joris 1995; Saraste and Pulkki 2000; Nathan and Ding 2010). The property of the apoptosis is the DNA fragmentation of 180- to 200-bp intervals between nucleosomes. This fragmentation, which produces a ladder pattern in agarose gel electrophoresis, may involve DNA fragments of only 50 kbp or may vary depending on the cell type (Montague and Cidlowski 1996). Translocation of phosphatidylserine into the plasma membrane is one of the significant alterations that occur during apoptosis, promoting recognition of apoptotic cells by the neighboring cells and macrophages (Vance and Steenberger 2005; Mourdjeva et al. 2005). Apoptosis is induced by several factors, which can be classified as: extracellular factors and intracellular factors depending on the conditions outside or inside the cells. Extracellular factors provoke apoptosis by death of receptors on the cell surface whereas intracellular factors trigger the mitochondrial activation. Cytochrome c is considered to have a central role in apoptosis because its release into the cytosol points out the irreversible stage of apoptosis (Kluck et al. 1997). Cytochrome c, together with apoptosis-inducing factors (AIFs), is released from the mitochondria into the

cytosol and binds to and activates a cytoplasmic protein Apaf-1 (apoptotic protease activating factor-1) by forming a complex named apoptosome. This complex activates procaspase-9 in active caspase-9. Active caspase-9, in turn, activates procaspase-3, which is one of the effector caspases (Li et al. 1997). Active caspase-3 inactivates the inhibitor of caspase-activated deoxyribonuclease (ICAD), thus liberating caspase-activated deoxyribonuclease (CAD) bound to ICAD, which results in chromatin condensation and oligonucleosomal DNA fragmentation, characteristic features of apoptosis (Enari et al. 1998). This mechanism is named caspase-dependent apoptosis, or intrinsic apoptosis whereas a caspase-independent pathway of apoptosis also exists. AIFs, released from the mitochondria induce caspase independent apoptosis, but the affected nuclease remains unknown (Hunot and Flavell 2001). Caspases are a group of enzymes also termed as cysteine proteases because they involve a cysteine residue in their active regions (Hardy et al. 2004). Caspases activate each other, resulting in a cascade of proteolytic reactions. They are divided in initiator caspases (caspase 2, 8, 9 and 10), and effector caspases (caspase 3, 6 and 7) (Slee et al. 2001; Chen and Wang 2002). The first ones transmit the death signal, generated by the apoptotic stimulus, to the second ones, which cleave the target proteins [e.g. proteins of cellular skeleton actin and fodrin, nuclear membrane protein laminin A, poly (ADP-ribose) polymerase (PARP), which is involved in DNA repair] and thus produce the morphological features of apoptosis. Activation of caspase cascade occurs by the release of cytochrome c to the cytosol and activation of procaspase-9, whereas caspases themselves also can lead to the release of cytochrome c to the cytosol.

The mitochondrial permeability transition

In the last decades the effects of various molecules and compounds on the intrinsic apoptosis have been studied. There are a lot of evidences that biogenic amines can participate in the regulation of apoptotic pathways by interacting with mitochondria (Toninello et al. 2004; 2006a; 2006b; Agostinelli et al. 2010). In particular, these amines are involved in the mitochondrial permeability transition (MPT) that is characterized by the opening of a channel, the transition pore (PTP), which permits nonspecific bi-directional traffic of solutes, having molecular mass less than 1500 Da, across the inner membrane, leading to swelling of the organelle and release of cytochrome c and AIF. This phenomenon is provoked by the presence of specific inductors and/or oxidative stress and with altered calcium homeostasis. Calcium ion is transported into the matrix by an electrophoretic uniport (specific for the uptake) and it is expelled by an

electroneutral antiport. The efflux occurs in exchange with two protons (H⁺) or two Na⁺ for every Ca²⁺ (Skulachev 1999). Cellular calcium homeostasis is maintained by these transporters; whereas, during MPT, Ca²⁺ is released from mitochondria provoking modifications on activity of several mitochondrial enzymes regulated by its concentrations. In this condition the mitochondrial inner membrane is no more completely impermeable to ions and small molecules and thus does not maintain the electrochemical gradient ($\Delta\mu_{H}^{+}$) with a consequent block of ATP synthesis.

The most known and studied inducer of MPT is Pi, that crosses the mitochondrial membrane as uncharged ortophosphoric acid (H_3PO_4), it dissociates in matrix and release $2H^+$ reducing the inner alkaline pH. This event augments the $\Delta\Psi$, with consequent increase in the accumulation of Ca²⁺. Other MPT inducers provoke the production of ROS in mitochondria, with consequent alteration of the redox state of several mitochondrial components, such as pyridine nucleotides and thiols. The inhibition of MPT involves molecules that interfere with calcium accumulation due to the action of inducers, or they act on the PTP structure preventing its opening, such as the immunosuppressant Cyclosporin A (CsA), ADP, ATP and bogkrekic acid (BKA) (Zoratti and Szabò 1995).

Depending on cell type, metabolic conditions, and their cytosolic concentrations biogenic amines act, in vivo, as promoting or protective agents of mitochondrialmediated apoptosis. With regard naturally occurring polyamines (putrescine, spermidine, spermine) are important inhibitors in vitro of MPT which is strictly connected with apoptosis. In particular, spermine can be considered as one of the most powerful physiological inhibitor agents because of its free radical scavenging action in isolated mitochondria (Sava et al. 2006), but also for its probable interaction with the anion groups present on the pore forming structures. Instead, agmatine, a dicationic amine at physiological pH, exhibits a dual effect in RLM: at low concentrations induces the MPT; this effect might be likely due to oxidation of agmatine by a mitochondrial amino oxidase with the production of hydrogen peroxide and, most probably, other reactive oxygen species (ROS). While, at high concentrations agmatine prevents the MPT. A possible explanation for this action is that even if the amine can produce ROS the amount of still unreacted molecule may act as their scavenger, by exhibiting a self-protection against the ROS produced by itself (Battaglia et al. 2007). It is to underline that in hepatocyte cultures agmatine behaves as an apoptosis inducer (Gardini et al. 2001).

Concerning the monoamines the studies reported in literature are very scarce. It has been demonstrated that in RLM tyramine is able to trigger the MPT in the presence of calcium. In fact, it induces MPT-associated events that are prevented

by MPT inhibitors (Marcocci et al. 2002). Other investigations refer to the effect of dopamine in RLM (Brenner-Lavie et al. 2008) but the results are not conclusive and no mechanism has been identified.

Aim of work

In the context of a research program directed at defining the factors modulating the mitochondrial permeability transition (MPT), the present study aimed to elucidate the role of some monoamines on this process. The MPT is a well studied phenomenon associated with an opening of the so-called permeability transition pore (PTP), that drives the bidirectional transport of ions and metabolites across the mitochondrial membrane(s). This process induces mitochondrial swelling with the subsequent rupture of membranes and release of cytochrome C and other proteins that may cause cellular death. It is well known that monoamines play an important role in various neurological processes and that neurons are monoamine producing cells, while mature hepatocytes do not normally produce this type of amino-groups-containing compounds. However recently several studies focus on the involvement of these amines, in particular serotonin, in liver regeneration and fibrosis, and dopamine that provides cell protection against various pathologic events.

In this study the action of these amines on MPT induction in isolated rat mitochondria from different organs and their possible implications is reported.

Materials and Methods

<u>Materials</u>

The fluorescent probes Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) was purchased by Molecular Probe, Inc. was dissolved in analytically pure DMSO and stored at -20°C. Fatty acid free Bovine Serum Albumin (BSA), Horseradish Peroxidase (E.C. 1.11.1.7), Serotonin, Tyramine and Dopamine were from Sigma. The monoclonal anti-phosphotyrosine antibody was provided by Millipore.

<u>Methods</u>

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), rat heart mitochondria (RHM), and rat brain mitochondria (RBM) were prepared with the following method.

The organs, liver, kidney, heart, and brain, were taken from Wistar rats of 180 gr 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).
- For RHM: 300 mM sucrose, 5 mM Hepes and 10 mM EDTA (ph 7.5)

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, RKM, and RHM, and at 11000 rpm (15900 g) for RBM, for 10 min.

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

- Liver, heart and kidney mitochondria precipitates were washed by a final centrifugation at 11000 rpm (15900 g) for 5 min, in medium without EGTA. Finally, they were resuspended in 2 ml of final medium and were 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 is based on the formation of a violet complex between rameic ion and amidic nitrogen of protein (biuret reaction).

The Gornall's solution contains:

- Rameic sulphate (CuSO₄ 5 H₂O) 1.5 g/l
- Sodium and potassium tartrate (NaKC₄H₄O₆ 4 H₂O) 6 g/l
- Sodium hydroxide (NaOH) 30 g/l

The solution is photosensible and is conserved at dark.

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

Standard medium for mitochondrial measurement

RBM, RHM, RKM, and RLM (1 mg protein/ml) were incubated in a water-jacketed cell at 20°C. The standard medium contained 200 mM sucrose, 10 mM HEPES (pH 7.4), 5 mM sodium succinate, 1 mM sodium phosphate, and 1.25 μ M rotenone. Variations and/or other additions are described in the specific experiments presented.

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⁺ permeable-selective 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) forming a pile, with an electrogenic force/power that results linear function of activity logarithm of ion to measure.

The membrane separates two solutions 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 produced by tetraphenylborate (TPB⁻). The membrane is a cationic membrane, because is permeable to TPP⁺.

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



Fig. 3. TPP⁺ selective electrode.

The inner and outer electrode potentials are 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 interliquid 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:

E₁ = ionoselective electrode potential

E₂ = outer reference electrode potential + interliquid junction potential

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

 ΔE_x = ionoselective membrane potential difference

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

 E_L = interliquid 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:

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

Where:

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

The electrode response is linear with the logarithm of TPP^+ concentration, with an increase of about 58 mV per ten units 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:

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

Where:

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

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

Determining the variation of electrode ΔE .

Considering the law of mass conservation:

$$V[TPP^+]_{out} + v[TPP^+]_{in} = V[TPP^+]_0$$
^[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 ($\approx 1 \, \mu$ l)

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

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

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

Where:

ΔE_0 = electrode potential difference before mitochondrial addition

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

The v value was calculated using the [¹⁴C]sucrose distribution (Palmieri e Klingenberg, 1979), and it corresponds to 1μ l/mg of mitochondrial proteins. Jensen et al. (1986), comparing the $\Delta\Psi$ value obtained measuring the ⁸⁶Rb and that measured with electrode, propose to correct the value adopting the following equation:

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

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

Before to proceed with the $\Delta\Psi$ measure, the electrode calibration is performed 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, but without mitochondria, adding TPP^+ and measuring the electrode variation. The final

concentration of TPP⁺ should not exceed 1-2 μ M, because an excessive amount of TPP⁺ can later depolarize the mitochondrial membrane.

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

After calibration, mitochondria were added to incubation medium, TPP⁺ enters the 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 $\Delta E - \Delta E_0$, and registered by the recorder as a deflection corresponding to the decrease of TPP⁺ concentration in the medium. The fitting of this potential difference variation in the equation [4] allows to obtain the $\Delta \Psi$ value.

Uptake of serotonin in mitochondria

Mitochondria were incubated in the standard medium in presence of radiolabeled serotonin (50 μ Ci/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 by mitochondria was counted in a specific scintillator (Liquid Scintillation Analyzer Packard 1500) after mixing with a 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:

anode: $4Ag + 4Cl^{-} \longrightarrow 4AgCl + 4e^{-}$ cathode: $O_2 + 4H^{+} + 4e^{-} \longrightarrow 2H_2O$ The overall result is that of a transfer of electrons from the cathode to the anode causing a current flow between the two electrodes which can be measured in an external circuit and drawn in a curve that measures the current intensity during the time. The electrical 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, were done by addition of 200 μ M ADP to the mitochondrial suspension. The ratio of oxygen uptake in state 3 (in presence of ADP) was divided by the rate of oxygen uptake in state 4 (in absence of ADP) to obtain the RCI. 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 for the hydrogen peroxide determination

The hydrogen peroxide produced by mitochondria is measured by MAO activity. The assay was based on the detection of hydrogen peroxide generated during substrate catabolism in a horseradish peroxidase (HRP) coupled reaction using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent, Molecular Probes). The mitochondrial protein extracts (50 μ g) were incubated in PBS with 40 μ M Amplex Red and 15 μ g/ml HRP. The reaction was started by the addition of 100 μ M tyramine, a MAO-A and MAO-B substrate. The fluorescence intensity was recorded at 37°C using a Perkin Elmer LS-50B fluorimeter at the 544/590 nm excitation/emission wavelengths. Parallel samples were run in the absence of a substrate to take into account the increase in fluorescence not due to MAO activity.

Redox state determination of sulhfydryl groups

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



After the specific incubation, the samples were centrifuged at 12000 rpm (11742 g) 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 wavelength. The sulfhydryl groups concentration was obtained by Lambert-Beer law, with ϵ =13600 M⁻¹cm⁻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 excitation and 462 nm of emission wavelengths respectively.

Mitochondrial swelling determination

Mitochondrial swelling occurs when solute(s) enter(s) in high quantity in the mitochondrial matrix, e.g. when mitochondrial permeability transition occurs, causing an increase in the osmotic pressure.

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

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

Detection of tyrosine phosphorylation of mitochondrial proteins

The polyacrylamide gel electrophoresis is one of the most widely used methods to separate a mixture of proteins and determine their molecular weight. Before loading on the gel, the samples were boiled for 5 minutes in the presence of β -

mercaptoethanol, which cleaves the disulfide bonds destabilizing the tertiary structure of proteins, and SDS. The dodecyl sulphate of sodium (SDS) is an ionic detergent that binds tightly to proteins and causes their denaturation. In the presence of an excess of SDS about 1.4 gr of detergent binds to mg ptotein, the protein providing thus a constant value of negative charge per mass unit. Therefore, in the electrophoresis courses, all SDS-protein complexes move towards the anode and, for molecular sieve properties of the gel, their mobility is inversely proportional to molecular weight. If standard proteins of known molecular weight are loaded in parallel to the samples, it is possible to determine the protein molecular weight of the samples. The SDS polyacrylamide gel is prepared following the method of Laemmli (Laemmli, 1970).

The electrophoretic plate is composed of two types of gel:

- layer of gels (stacking gel) at pH 6.8, which serves to concentrate the protein sample;
- separation gel (running gel) at pH 8.8, through which the real separation of proteins takes place.

This plate, of size of 8x10 cm, was fixed at a special unit (SE 250 Hoefer Mighty Small-SCIENTIFIC INSTRUMENTS). The duration of electrophoresis is approximately 1.5 h under an electrical current of 40 mA / gel.

The transfer of band proteins from electrophoretic polyacrylamide gels to a nitrocellulose membrane, was performed by applying at the appropriate unit a current of 350 mA for 2 hours. The buffer used for electrophoresis is composed of:

- 25 mM Tris
- 192 mM glycine
- 20% methanol
- 0.1% SDS with a final pH of 8.0.

After blotting the membrane was allowed to saturate for 30 min in the following buffer:

- 50 mM Tris-HCl pH 7.5
- 150 mM NaCl
- 3% BSA.

The nitrocellular membrane was then incubated overnight at 4 °C with an appropriate antibody diluted in the following buffer (buffer C):

- 50 mM Tris-HCl pH 7.5
- 150 mM NaCl
- 1% BSA

supplemented with 0.02% NaN₃.

3 washes of 10 minutes each were performed, at room temperature, with buffer C supplemented with 0.1% Tween (washing buffer). At this point the membrane was incubated for 30 min with an anti-IgG, diluted in buffer C, obtained against the animal species immunized for the first antibody. After 3 additional washes, the antibody detection with the ECL system ("Enhanced Chemi Luminescence") was performed. The reagents, in contact with the membrane, corresponding to the antigen-antibody complex give rise to a reaction of chemiluminescence. Above the membrane was placed for a few seconds, an autoradiographic plate, which is impressed by the light emission.

Phosphorylation assay

Tyrosine phosphorylation was assayed by incubating 1 mg of brain mitochondria at 20° C for 10 min in 1 ml of standard medium in the presence of several phosphatase inhibitors. After incubation, samples were analyzed by SDS–PAGE, the separated proteins were immediately electrophoretically transferred to nitrocellulose membranes, treated with anti-phosphotyrosine antibody, followed by secondary peroxidase-conjugate antibody and detected by the enhanced chemi-luminescence technique (ECL, Amersham Pharmacia Biotech).

Effects of monoamines on the mitochondrial permeability transition

Biogenic amines, wich are the object of the present study, such as serotonin, dopamine and tyramine, play key roles in neurotransmission and other signaling functions. They are relatively small in size and contain a protonated amino group or a permanently charged ammonium moiety. They can act as neurotransmitters to elicit various physiologic responses, and they all have various other sites of action throughout the body (Zeisberger 1998; Toninello et al. 2004). As recently demonstrated, platelet-derived serotonin mediates liver regeneration (Lesurtel et al 2006) after partial hepatectomy in mice. Furthermore, the hepatic stellate cell (HSC) is recognized as one of the key mediators in the progression of hepatic fibrosis (Friedman 2000). A great deal of attention has recently focused in the process of activated HSC apoptosis because stimulation of this process promotes accelerated rate of recovery from rat liver fibrosis and serotonin has been shown to influence the proliferation and apoptosis of activated HSCs (Friedman 2000; Wright et al. 2001; Ruddell et al. 2006).

Many tissues, such as lung, spleen, liver, brain and the enterochromaffin cells of the gut, take up serotonin from the circulating extracellular fluid even though they contain locally produced endogenous serotonin (Sirek and Sirek 1970).

Furthermore, experimental evidences from cell culture studies, show that dopamine provides cell protection against various pathologic stimuli (Miura et al. 1998; Brinkkoetter et al. 2006; Rudic et al. 2009) and also confers protection during ischemic cold storage in kidneys (Liu et al. 2007) and livers (Koetting et al. 2010).

The degradation of these molecules is catalyzed, as mentioned above, by monoamino oxidases (MAOs) A and B that are mitochondrial membrane bound isoenzymes able to give rise an oxidative deamination. The chemical reaction catalyzed by MAOs consists in the degradation of monoamines into the corresponding aldehydes and hydrogen peroxide.

$RCH_2NH_2 + O_2 + H_2O \longrightarrow RHCO + NH_3 + H_2O_2$

Aldehydes are then oxidized into acids by aldehyde dehydrogenase or converted into alcohols or glycols by aldehyde reductase. In particular, hydrogen peroxide can trigger the production of other reactive oxygen species and induction of mitochondrial damage and neuronal apoptosis (Bortolato et al. 2008).

As monoamines may be metabolized by MAOs, a first important consideration to take into account refers to the possible effects of monoamines, or their reaction products, on the bioenergetic parameters in isolated mitochondria.

Results

Mitochondrial inner membrane integrity provides insulating properties and a correct electron flux along the respiratory chain that are peculiar characteristics of energy-transducing membranes. This integrity can be estimated by the membrane potential $\Delta \Psi$ and respiratory control index (RCI) determination. The first is a value that actually indicates the membrane insulating capacity. $\Delta \Psi$ is one of two factors (the other being ΔpH) which constitute the electrochemical gradient ($\Delta \mu_{H}^{+}$). During ATP synthesis, a drop in $\Delta \mu_{H}^{+}$, a peculiar characteristic of the energy trasducing membranes, is experimentally appreciable by a transient decrease in $\Delta \Psi$. While, the latter indicates the coupling between oxygen consumption and ATP synthesis. In order to evaluate implications of monoamines on the mitochondrial function it is necessary to assess their effects on these two bioenergetic parameters. Figure 4 shows that RLM (A) and RBM (B), energized by the oxidation of succinate in the presence of rotenone, and incubated under the adopted experimental conditions with 100 μM serotonin and dopamine, don't exhibit any significant alterations on the oxygen consumption.



Fig. 4 Effect of monoamines on the oxygen consumption in RLM (A) and RBM (B).

Mitochondria were incubated in standard medium, as described in Materials and Methods section. Serotonin (SER) and dopamine (DA) concentrations are indicated at side of traces. Results are representative of four experiments.

The tables in Fig. 4 report the calculation of RCI and ADP/O ratio. Both monoamines maintain the oxidative phosphorylation parameters near normal levels.

Serotonin and dopamine do not cause alterations on $\Delta \Psi$ in both types of mitochondria (Fig. 5). It is also worthy to evidence that addition of a limited amount of ADP induces the same transient drop in $\Delta \Psi$ in the absence or presence of the monoamines.



Fig. 5 Effect of monoamines on membrane potential ($\Delta\Psi$) in RLM (A), and RBM (B).

Mitochondria were incubated in standard medium, as described in Materials and Methods section. Serotonin (SER), dopamine (DA) and ADP concentrations are indicated at side of traces. Results are representative of four experiments. ΔE = electrode potential
These results suggest that, most probably, the reactive oxygen species produced by monoamino oxidase activity are not in sufficient amount to overcome the safety systems present in mitochondria by which their membranes remain intact and functioning. Almost identical results have been observed by treating rat mitochondria extracted from kidney (RKM) and heart (RHM) with the above amines (not shown).

When treated with supraphysiological Ca²⁺ concentration (50 μ M), RLM exhibit a consistent decrease in $\Delta\Psi$ from 180 mV to 140 mV in 10 minutes of incubation (Fig. 6A). This event is paralleled by an apparent optical absorbance decrease of about 0.200 arbitrary units/10 min that is indicative of mitochondrial colloid osmotic swelling occurrence (Fig. 6B).



Fig. 6 Effect of monoamines on membrane potential ($\Delta\Psi$) (A), and mitochondrial swelling (B) induced by Ca²⁺ in RLM.

Mitochondria were incubated in standard medium, as described in Materials and Methods section, supplemented with 50 μ M Ca²⁺ except when otherwise indicated. When reported the concentration of added monoamines was 100 μ M. Downward deflections indicate mitochondrial swelling (B). 2 μ M TPP⁺ was used for $\Delta\Psi$ measurements. Results are representative of five experiments. ΔE is the electrode potential (A).

In the presence of monoamines the $\Delta \Psi$ almost completely collapses (Fig. 6A) accompanied by a further marked absorbance decrease suggesting an increment of mitochondrial swelling.

The $\Delta\Psi$ collapse and the amplification of swelling are significantly inhibited by the typical MPT inhibitors cyclosporine A (CsA), ADP, and bongkrekic (BKA) (Figs. 7A and 7B).





RLM were incubated in standard medium, as described in Materials and Methods section, supplemented with 50 μ M Ca²⁺ except when otherwise indicated. Monoamine concentration was 100 μ M, while: 1 μ M CsA, 500 μ M ADP and 5 μ M BKA were present when indicated. Results are representative of four experiments. Δ E= electrode potential.

To investigate the cause of this amplification both in $\Delta\Psi$ drop and swelling it has been hypothesized that monoamines could induce an oxidative stress. Fig. 8 reports the effects of antioxidant agents butyl-hydroxytoluene (BHT) and dithioerythrytol (DTE), the alkylating agent N-ethylmaleimide (NEM) and the SHreagent 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) on $\Delta\Psi$ collapse (A) and on mitochondrial swelling (B) induced by monoamines plus Ca²⁺.





RLM were incubated in standard medium, as described in Materials and Methods section, supplemented with 50 μ M Ca²⁺ except when otherwise indicated. Serotonin (SER), dopamine (DA), and tyramine (TYR) were 100 μ M, while: 10 μ M NEM, 25 μ M BHT, 200 μ M DTNB and 3mM DTE were present when indicated. Results are representative of four experiments.

As shown in Fig. 8 all the above agents are able to completely prevent all these effects, thus indicating that the monoamines behave as amplifiers of the MPT

induced by Ca^{2+} and that these effects are likely consequent to an oxidative stress.

Since monoamines can provoke oxidative stress as a result of MAOs activity, the effect of toloxatone (TXT), a specific inhibitor of MAO-A, that is the isoform that metabolizes serotonin, and catalase, has been tested. The results reported in Fig. 9, besides to demonstrate that serotonin increases the extent of swelling induced by Ca^{2+} (see also Figs. 7, 8), also show that (TXT) counteracts this amplification while it is ineffective on the swelling induced by Ca^{2+} alone. The same effect is also displayed by catalase suggesting that H_2O_2 is the responsible agent that causes this amplification.



Fig. 9 Effect of MAO inhibitor and catalase on swelling of RLM induced by Ca^{2+} plus serotonin.

RLM were incubated in standard conditions described in Materials and Methods section, supplemented with 50 μ M Ca²⁺ except when otherwise indicated. When reported 100 μ M serotonin (SER), 100 μ M toloxatone, and 1000 U catalase were present. Results are representative of four experiments.

The results obtained with TXT and catalase confirm that the monoamine effects in amplifying the RLM swelling are due to an oxidative stress caused by oxidation products generated by MAO. The conclusions that monoamines induce an oxidative stress are consistent with the results of Fig. 10 showing their effect on the redox level of mitochondrial thiol groups.

In fact, besides Ca^{2+} , also monoamines themselves, induce the decrease of sulfhydryl groups content in RLM although with less effectiveness than the cation. Incubation of monoamines plus Ca^{2+} further decreases the level of SH content in agreement with the PTP opening.



Fig. 10 Effect of monoamines and calcium on the redox state of sulfhydryl groups in RLM.

Incubation conditions as described in Material and Methods section, supplemented with 50 μ M Ca²⁺, except when otherwise indicated, and 100 μ M monoamines where reported. The levels of sulfhydryl groups were measured after 12 min incubation and their total content was considered as 100 %. Values are the means ± SD of five experiments.**p* < 0.05

The analysis of the pyridine nucleotide oxidation gave similar results, and Fig. 11 reports the effects of Ca^{2+} and monoamines on this process in RLM.



Fig. 11 Effect of monoamines and calcium on the pyridine nucleotides reduced state in RLM. Incubation conditions as described in Material and Methods section, supplemented with 50 μ M Ca²⁺ except when otherwise indicated and 100 μ M monoamines where reported. Results are representative of four experiments.

This figure shows that Ca^{2+} by itself induces a significant oxidation of these nucleotides, while monoamines alone are not effective. When Ca^{2+} and monoamines are supplemented together the oxidation is strongly enhanced.

Generally, the opening of PTP by Ca^{2+} is related to production of H_2O_2 and alteration of the redox state of several mitochondrial components, therefore we detected the production of hydrogen peroxide induced by the mentioned above compounds. It was not possible, however, use the serotonin and dopamine because of their interference with the detection method. We then used tyramine that does not have these problems and the Fig. 12 shows that this amine produces high levels of this reactive oxygen species.



Fig. 12 Effect of monoamine and calcium on the hydrogen peroxide production in RLM.

Incubation conditions as described in Material and Methods section, supplemented with $50 \mu M \text{ Ca}^{2+}$, except when otherwise indicated, and $100 \mu M$ monoamines where reported. Values are the means ± SD of five experiments.

RBM incubated with 1 mM sodium phosphate and 50 μ M Ca²⁺, that is the same ion concentration used for experiments performed with RLM, undergo a colloidosmotic swelling of much lesser extent than RLM, therefore 100 μ M Ca²⁺ was generally used in the following experiments even if RBM, energized by succinate plus rotenone and incubated with this concentration undergo a colloid-osmotic swelling less marked than that observed in RLM (Fig. 13B). RBM, in control conditions, exhibit a $\Delta\Psi$ value of about 160 mV. When Ca²⁺ together with phosphate are present, a slight decrease of $\Delta\Psi$ is observed, while the monoamines, as in the swelling experiments, do not display any significant effect (Fig. 13A).



Fig. 13 Effect of monoamines on membrane potential ($\Delta\Psi$) (A), and mitochondrial swelling (B) induced by Ca²⁺ in RBM.

Mitochondria were incubated in standard medium, as described in Materials and Methods section, supplemented with 100 μ M Ca²⁺ except when otherwise indicated. When reported the concentration of added monoamines was 100 μ M. Downward deflections indicate mitochondrial swelling (B). 2 μ M TPP⁺ was used for $\Delta\Psi$ measurements. Results are representative of four experiments. ΔE is the electrode potential (A).

The observations that monoamines do not increase the collapse of $\Delta \Psi$ provoked by Ca²⁺ and do not amplify the Ca²⁺-induced swelling, led us to hypothesize that monoamines were not able to oxidize the mitochondrial structures involved in the MPT, in particular the thiol groups.

The results obtained in Fig. 14 show that Ca^{2+} and all the tested monoamines, incubated alone, induce a decrease of the SH content of about 10%. In addition, unlike RLM, when the monoamines are incubated in RBM in the presence of Ca^{2+} , no further decrease in SH levels is found. In conclusion these results demonstrate that in RBM the monoamines are unable to increase the oxidative stress induced by Ca^{2+} and, consequently are ineffective in amplifying the MPT.



Fig. 14 Effects of calcium and monoamines on the redox state of sulfhydryl groups in RBM. RBM were incubated in standard medium, as described in Materials and Methods section, supplemented with 100 μ M Ca²⁺ except when otherwise indicated. 100 μ M monoamines present when reported. The levels of sulfhydryl groups were measured after 12 min incubation and their total content was considered as 100%. Values are the means ± SD of five experiments. .*p < 0.05

These observations stimulated the interest to investigate about a possible different mechanism of MPT in RBM at variance to that well known in RLM.

The analysis of the effects of MPT specific inhibitors in RBM shows that CsA almost completely blocks the swelling induced by Ca²⁺, while ADP and BKA exhibit only a partial and negligible protection, respectively (Fig. 15).



Fig. 15 Effect of MPT inhibitors on mitochondrial swelling induced by Ca²⁺ in RBM. RBM were incubated in standard medium, as described in Materials and Methods section, supplemented with 50 μ M Ca²⁺ except when otherwise indicated. 1 μ M CsA, 500 μ M ADP and 5 μ M BKA were present when indicated. Results are representative of four experiments.

Furthermore we used the antioxidant agents BHT and DTE, as well as TXT and catalase, and, as shown in Fig. 16, it is clear that they are almost, or completely ineffective, in inhibiting the swelling induced by Ca²⁺, whereas the SH-reagents, DTNB and NEM, are partial inhibitors of this process.



Fig. 16 Effect of antioxidant agents on mitochondrial swelling induced by Ca^{2+} in RBM. RBM were incubated in standard medium, as described in Materials and Methods section, supplemented with 100 μ M Ca^{2+} . When indicated: 10 μ M NEM, 25 μ M BHT, 200 μ M DTNB and 3 mM DTE, 100 μ M toloxatone, and 1000 U catalase were present. Results are representative of four experiments.

In order to better understand the opening mechanism of the PTP in RBM we tested DTE, BHT and NEM on the redox state of sulfhydryl groups. As shown in Fig. 17, Ca²⁺ induces a decrease in the content of reduced thiols, most likely corresponding to the formation of oxidized dithiol groups, whereas DTE and BHT counteract the cation effects. Instead, addition of NEM does not bring about any protection on this oxidation.





RBM were incubated in standard medium, as described in Materials and Methods section, supplemented with 100 μ M Ca²⁺. When indicated: 10 μ M NEM, 25 μ M BHT and 3 mM DTE. The levels of sulfhydryl groups were measured after 12 min incubation and their total content was considered as 100%. Values are the means ± SD of five experiments. **p* < 0.05

 Ca^{2+} alone also provokes a certain oxidation of the pyridine nucleotides (Fig. 18), while monoamines are not effective; moreover the oxidation of pyridine nucleotides by Ca^{2+} is not increased when the cation is incubated together the monoamines.



Fig. 18 Effect of monoamines and calcium on the pyridine nucleotides in RBM.

Incubation conditions as described in Material and Methods section, supplemented with 100 μ M Ca²⁺, except when otherwise indicated, and 100 μ M monoamines were present where reported. Results are representative of four experiments.

generation with SER or DA by means of the peroxidase method. Indirect measurements of H_2O_2 generation using a Clark electrode have shown identical results among TYR, SER and DA (results not reported).

The H_2O_2 production by TYR is similar to that formed by RLM, although to lesser amount, whereas Ca²⁺ is almost completely ineffective in producing this ROS species in this type of mitochondria (Fig. 19).



Fig. 19 Effect of monoamine and calcium on the hydrogen peroxide production in RBM. Incubation conditions as described in Material and Methods section, supplemented with 100 μ M Ca²⁺ except when otherwise indicated. When reported: 100 μ M tyramine was present. Values are the means ± SD of five experiments.

Indeed, this consistent amount of hydrogen peroxide produced by tyramine oxidation is not enhanced by the presence of Ca^{2+} .

Discussion

Several studies concerning the determination of MPT and its molecular mechanism of induction have been performed in RLM. In particular, this type of mitochondria has been used as a target where the biogenic amines behaved as inhibitors or inducers of the above phenomenon. These studies considered principally the action of the natural polyamines (Toninello et al. 2004; Sava et al. 2006), but also that of the diamines, in particular agmatine (Battaglia et al. 2007) and the propargylamines (Marcocci et al. 2002). As far as the monoamines are concerned, only tyramine and dopamine have been investigated as MPT inducers in RLM (Marcocci et al. 2002; Brenner-Lavie et al. 2008).

This part of thesis deals with the different effects exhibited by the monoamines serotonin, tyramine, and dopamine on the Ca²⁺-dependent MPT induction in RLM, RHM, RKM and RBM.

The experiments of figures 6, 7 and 8, show the ability of monoamines to amplify in significative manner this phenomenon triggered by Ca²⁺ in RLM, through an oxidative stress consequent to their oxidation. Monoamines induce the mitochondrial swelling and $\Delta \Psi$ collapse in the following order: serotonin > tyramine > dopamine. The demonstration that monoamines amplify the PTP opening in RLM is given by the observation that typical inhibitors of this phenomenon, such as CsA, ADP, and BKA, completely prevent it (Fig. 7A and 7B). Furthermore this process is attributable to an oxidative stress as the antioxidant agents DTE and BHT, as well as the SH-reagent DTNB and the alkylating agent NEM, significantly inhibit it (Figs. 8A and 8B). It is well known that a mitochondrial MAO, located on the external side of outer membrane, releases hydrogen peroxide as a by-product of monoamine oxidation. The observation that both TXT and catalase partially inhibit the RLM swelling (Fig. 9) suggests that the swelling amplification induced by serotonin is due to the oxidative stress caused by MAO-mediated generation of H_2O_2 . This is also strongly supported by the fact that TXT and catalase reduce the swelling to almost the same extent induced by Ca²⁺ alone. A further confirmation that the opening and the amplification of PTP depend on oxidative stress is showed in Figs. 10, and 11 where Ca^{2+} or monoamines can each induce the oxidation of sulfhydryl groups and pyridine nucleotides in parallel with H₂O₂ generation (Fig. 12).

The lack of correlation between the oxidation of sulfhydryl groups, and pyridine nucleotides, and the tyramine-produced high amounts of H_2O_2 , might be due to the fact that the H_2O_2 produced may interact with other targets. The thiol and pyridine nucleotide oxidation observed in the presence of Ca²⁺ is most probably due to the interaction of the cation with the membrane cardiolipins that causes

membrane disorganization and consequent alteration of ubiquinone mobility (Grijalba et al. 1999). This process would favour the generation of the semiquinone radical and, consequently, the superoxide anion by interacting with oxygen. Then, superoxide anion would form the hydrogen peroxide production by the activity of superoxide dismutase (Agostinelli et al. 2004). Furthermore H_2O_2 , by interacting with Fe²⁺ of respiratory chain complexes would generate the highly reactive hydroxyl radical by means of the Fenton reaction, most likely responsible of thiol and pyridine oxidation (Sava et al. 2006).

However, the oxidation of thiols (Fig. 10) and pyridine nucleotides (Fig. 11), induced by the monoamines is likely due to the generation of H_2O_2 by MAO activity (Fig. 12). In literature it is generally reported that the PTP opening requires the interaction of Ca^{2+} with a specific site located in the AdNT (Halestrap and Brenner 2003) or, as recently proposed, in the phosphate carrier (Leung et al. 2008), with the oxidation of two specific cysteine SH groups located in these carriers. As the presence of Ca^{2+} is necessary for triggering the phenomenon, we conclude that monoamines alone are not able to open the PTP, but they potentiate the effect of Ca^{2+} in RLM by means of further production of H_2O_2 as the result of an increased MAO activity.

In RBM, Ca^{2+} induces mitochondrial swelling and $\Delta\Psi$ drop, even if to a lesser extent than that observed in RLM (Figs. 6 and 13). This result suggest that MPT is induced by Ca^{2+} by a mechanism apparently similar to that proposed for RLM. In fact, the well-established inhibitor CsA also prevents this process in this mitochondrial type (Figs. 7 and 15). However, the monoamines, under our experimental conditions, do not cause significant alterations in the swelling and $\Delta\Psi$ drop induced by Ca^{2+} (Fig. 13).

Furthermore, apart from CsA, all the other inhibitors showed weak capacity to prevent the Ca²⁺-induced PTP. In fact, ADP, DTNB and NEM exhibited only partial protection, while BKA, DTE and BHT were completely ineffective (Figs. 15 and 16). This would mean that the classic mechanism proposed for RLM is not valid for RBM. Indeed, the partial or complete lack of protection by the antioxidant agents, as well as the ineffectiveness exhibited by the monoamines, suggests that in RBM the opening of the PTP is not necessary responsive to oxidative stress induced by hydrogen peroxide. In this regard the slight decrease in SH content induced by Ca²⁺ and monoamines themselves does not influence MPT induction and does not appear synergic in RBM (Fig. 14). The ineffectiveness exhibited by ADP and BKA suggests the involvement of a protein other than AdNT, even if CsA completely blocks the PTP opening and this confirms the involvement of cyclophilin in this process in both RLM and RBM. It is also important to note that the thiol oxidation by the monoamines is not

accompanied by a parallel oxidation of pyridine nucleotides (Figs. 14 and 18), suggesting that the oxidation of the above compounds is not correlated with one another. The further observation that monoamines are able to produce a consistent amount of H_2O_2 far higher than that produced by Ca^{2+} , and that the presence of calcium does not significantly change the H_2O_2 generation by monoamines (Fig. 19), can be explained by the observed slight oxidation of thiols and pyridine nucleotides in the presence of Ca²⁺ that, most probably, consumes the H₂O₂ produced in this condition leaving almost undetectable amounts of this compound. In conclusion, the results reported in this first part demonstrate a significant difference between RLM and RBM in the mechanism of MPT induction. In fact, at variance with RLM (and also RKM and RHM), RBM do not require H₂O₂-dependent oxidative stress to undergo this process. This observation could modify the general opinion concerning the mechanism triggered by Ca^{2+} in the presence of phosphate. Most probably H_2O_2 indirectly produced by Ca²⁺, or by monoamine oxidation, is able to oxidize specific sulfhydryl groups, but not the critical thiols that take part in the opening of PTP in RBM. The different behaviour of the two types of mitochondria may be related to the fact that they belong to different tissues with specific physiological processes and the peculiar adaptation of the metabolic networks to the different tissue functions. It is important to remind that neurons produce monoamines while mature hepatocytes do not. However, under proinflammatory or stress situations monoamines can be produced by immune cells infiltrated in liver. The different expression/properties of the MAO isoenzymes between liver and brain (Richardson 1993; Remaury et al. 1999; Youdim et al. 2001) could be at the base of the observed different actions.

So it can be argued, even if it is too early to draw definitive conclusions, that the roles of monoamines reported here may be correlated with MAO-dependent neuropathologies, such as Parkinson's disease (Richardson 1993; Remaury et al. 1999; Youdim et al. 2001; Rajput et al. 2008), and liver pathologies such as cirrhosis and steatohepatitis (Butterworth 2000; Nocito et al. 2007).

Uptake of monoamines in mitochondria

In order to shed some light on the mechanisms by which polyamines seem to be involved in the phenomenon of MPT in RLM, RKM and RHM, but not in RBM even if they can produce an oxidative stress in all types of mitochondria, we thought worth to investigate whether they may be transported in mitochondria.

It has already been observed that polyamine uptake is the sum of two processes: membrane binding (Marcocci et al. 2002; Di Noto et al.1996; Dalla Via et al. 1996) and electrophoretic matrix transport by a specific uniporter (Dalla Via et al. 1999; Toninello et al. 1988; Toninello et al 1992). This transporter is a common channel for all three natural polyamines that are transported bidirectionally across the inner membrane of liver mitochondria, with a saturable system, after the binding at two distinct binding sites in the membrane.

The transport of the diamine agmatine involves, most probably, a specific channel (gated pore) in liver and kidney mitochondria (Salvi et al. 2006), in particular it was found that mitochondrial membranes possess two agmatine binding sites S1 and S2, both with monocoordination. S1 is inhibited by idazoxan and these results suggest that it could correspond to the imidazoline receptor I_2 , that is a constitutively present 60-kDa protein localized on mitochondrial membranes and constituting a MAO domain. This suggests the involvement of I_2 receptors in the enzymatic activity control. Furthermore, agmatine can inhibit MAO activity and this effect could reasonably be mediated by its binding on these receptors (Toninello et al. 2009). In brain mitochondria the extent of initial binding is higher, whereas total accumulation is lower, than that in liver. The agmatine transporter is shared with the divalent putrescine and idazoxan markedly inhibits agmatine accumulation (but not binding). The transporter is a channel showing some characteristics similar to those of the imidazoline I₂ receptor and the sharing with the polyamine transporter (Battaglia et al. 2010). Monoamines, in particular serotonin and dopamine, are involved in a variety of neurophysiological processes. Serotonin is transported in nerve endings and platelets by very similar transport systems which are tightly regulated by their phosphorylated state (Zarpellon et al. 2008), and the cellular redox state. Dopamine, besides of its well known role of brain neurotransmitter, exerts specific functions at the periphery, in particular at the level of the cardiovascular system and kidney. In fact it modulates blood pressure, sodium balance, and renal functions through an independent peripheral dopaminergic system. It exerts its actions by a class of cell surface receptors belonging to the rhodopsinlike family of G-protein coupled receptors (Tayebati et al 2011). Considering that serotonin is present not only in cellular granules but also in cytoplasm (Sirek and Sirek 1970), and that it can be oxidized by the mitochondrial monoamines oxidases (MAOs), its possible transport in mitochondria, has been investigated.

Results

Under our experimental conditions about 30 nmol of serotonin/mg prot are almost instantaneously taken up by energized RLM, incubated in standard medium as described in Material and Methods section. Then, a gradual timedependent uptake is observable reaching a plateau in 30 min of incubation after an accumulation of about 15 nmol/mg prot (Fig. 20). Similar results are also obtained with RHM, RKM and RBM (not shown), but we decided to further investigate this process only in RLM.

A marked inhibitory effect on serotonin uptake is observed in the presence of FCCP (carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone), an uncoupler of mitochondrial respiration which provokes the collapse of $\Delta\Psi$ (Fig. 20). A similar effect has been also observed with antimycin A, an inhibitor of the respiratory chain (result not reported). Thus, serotonin uptake seems to depend on the energized state of mitochondrial membrane, suggesting the presence of an electrophoretic transport mechanism.





RLM were incubated in standard medium, as described in Materials and Methods section, with 1 mM [¹⁴C]serotonin (50 μ Ci/mmol). When present: 0.1 μ g/ml FCCP. Dotted lines and empty circles on ordinate axis indicate the extrapolation of serotonin binding at zero-time. Values are the means ± SD of five experiments. Inset: determination of mitochondrial membrane potential ($\Delta\Psi$). Δ E= electrode potential.

To further clarify the role of an energy-depence of this transport the action of some ionophores having different effects on $\Delta\Psi$ has been evaluated in comparison with that of FCCP. The results of Fig. 21 show that nigericin, which increases at maximum values $\Delta\Psi$ (Fig. 21, inset), induces a sudden uptake of serotonin followed by a gradual release. On the contrary, valinomycin plus K⁺, which completely collapses $\Delta\Psi$, induces a loss of accumulated serotonin. This effect is almost identical to that of FCCP. These results clearly demonstrate that serotonin, besides a rapid binding to the membrane, is transported in mitochondria by an energy dependent mechanism driven by $\Delta\Psi$.





RLM were incubated in standard medium, as described in Materials and Methods section, with 1 mM [¹⁴C]serotonin (50 μ Ci/mmol). When present: 0.3 μ g/ml nigericin, 0.3 μ g/ml valinomycin, and 0.1 μ g/ml FCCP. Dotted lines and empty circles on ordinate axis indicate the extrapolation of serotonin binding at zero-time. Values are the means ± SD of five experiments. Inset: determination of mitochondrial membrane potential ($\Delta\Psi$). Δ E= electrode potential.

In contrast to the effect of valinomycin, the addition of nigericin provokes an increase of the initial rate of serotonin uptake. This effect does not persist during

the incubation time most probably for the intense acidification consequent to the addition of nigericin to mitochondria.

As serotonin is oxidized by MAO we tested the effects of MAO inhibitors in order to verify if this enzyme is implicated in the monoamine uptake.

In the presence of clorgyline, a propargylamine well known inhibitor of MAO activity, serotonin uptake is strongly inhibited (Fig. 22). Since I₂ imidazoline receptor is present on mitochondrial membrane in both MAO-A and -B (Tesson et al. 1995) we also tested the effect of its antagonist idazoxan on serotonin uptake. As observable in Fig. 22 idazoxan decreases the initial membrane binding of serotonin and inhibits its net transport, thus further supporting a possible involvement of MAO in serotonin uptake.



Fig 22 Serotonin uptake by RLM: effect of idazoxan and clorgyline on serotonin uptake in RLM.

RLM were incubated in standard medium, as described in Materials and Methods section, with 1 mM [¹⁴C]serotonin (50 μ Ci/mmol). When indicated: 50 μ M clorgyline and 200 μ M idazoxan were present. Dotted lines and empty circles on ordinate axis indicate the extrapolation of serotonin binding at zero-time. Values are the means ± SD of five experiments. Inset: determination of mitochondrial membrane potential ($\Delta\Psi$). ΔE = electrode potential.

AS serotonin is oxidized by MAO with the production of H_2O_2 and the corresponding aldehyde, the observation that MAO inhibitors reduce the serotonin uptake leads to hypothesize that the enzyme may regulate this

accumulation since the transported species is not the original monoamine but its derived aldehyde. However it is also to consider that the transport mechanism seems to be electrophoretic therefore if we consider the molecular structure of both serotonin and its aldehyde it is difficult to propose a more active electrophoretic transport for the aldehyde. Instead another possibility is that the transport system is linked to MAO (possibly to the I₂ receptor) and that the binding of clorgyline and idazoxan might induce a conformational change of the enzyme thus affecting the transport system.

Considering that the optimal pH for MAO activity is about 9, we tested the serotonin uptake at different pH values in order to determine if the pH gradient affected and correlated with the amounts of accumulated neurotransmitter.

The results of Fig. 23 show that serotonin uptake in RLM incubated in medium at different pH: 6.5 and 8, was not significantly change in comparison with the uptake measured at the usual pH (7.4) adopted in our experiments.

This observation could support the hypothesis that the conformation of MAO is more important of its activity for serotonin transport.





RLM were incubated in 3 standard mediums at pH 6.5, 7.4 and 8, respectively as described in Materials and Methods section, with 1 mM [¹⁴C]serotonin (50 μ Ci/mmol). Dotted lines and empty circles on ordinate axis indicate the extrapolation of serotonin binding at zero-time. Values are the means ± SD of five experiments. Inset: determination of mitochondrial membrane potential ($\Delta\Psi$). ΔE = electrode potential.

The experiment of Fig. 24 was performed in order to clarify the localization of the consistent amount of serotonin apparently taken up by RLM at the zero-time. Mitochondria were incubated in the presence of 100 μ M labeled serotonin, then after 1 min 10mM unlabelled serotonin was added.



Fig 24 Serotonin uptake by RLM.

RLM were incubated in standard medium, as described in Materials and Methods section, with 1 mM [¹⁴C]serotonin (50 μ Ci/mmol). 10 mM serotonin was added 1 minute later the beginning of incubation. Dottes lines and empty circles on ordinate axis indicate the extrapolation of serotonin binding at zero-time. Values are the means ± SD of five experiments.

The result clearly demonstrates that the added 10 mM unlabelled serotonin detaches a consistent amount of $[^{14}C]$ serotonin (about 15nmol/mg protein) from mitochondria. However this result also evidences that a significant amount of amine is internalized in RLM after 1 min incubation.

Discussion

Unlike the effects of polyamines, those of monoamines on mitochondrial functions are very little known. On the basis of the actions of monoamines on the induction of MPT we have studied the possible transport of these amines in mitochondria of various organs such as liver, heart, kidney and brain. In particular, we have performed experiments in order to characterize the transport of the monoamine serotonin in RLM, as little has been done in this model with this important neurotransmitter.

Here it is reported the evidence that serotonin is able to bind to mitochondrial membranes and taken up into the matrix space. This binding is particular high if compared with that of the natural polyamines (Di Noto et al. 1996; Dalla Via et al 1999) and agmatine (Salvi et al. 2006) and it is slightly affected by clorgyline (Fig. 20) whereas it is unaffected by idazoxan and de-energizing agent FCCP, and ionophores nigericin, and valinomycin (Figs. 20 and 21).

The observation that addition of 10 mM unlabelled serotonin one min after the uptake of the radioactive amine induces a partial loss of it from mitochondria, supports the hypothesis that at the beginning of incubation, besides a membrane binding, a consistent amount of serotonin may be internalized. Furthermore, by considering the ineffectiveness of the above mentioned agents, this initial transport does not seem to be energy-dependent. A slower time-dependent serotonin uptake follows its membrane binding and it seems to depend on mitochondrial energization most probably being electrophoretic in nature (Fig. 20).

In fact, in the presence of valinomycin or FCCP, responsible of the collapse of $\Delta \Psi$, the time-dependent transport is markedly inhibited, on the contrary, in the presence of nigericin, which increases $\Delta \Psi$, the uptake is strongly enhanced. This second step of uptake exhibits a mechanism similar to that of polyamines.

As above described a gradual release of serotonin follows its uptake after nigericin addition. The explanation is that the strong acidification due to the entry of H^+ in exchange with K^+ induced by the ionophore damages the mitochondrion. This effect is not observable with the polyamines because of their polycationic structure they are able to maintain intact the mitochondrial membranes. Instead, the gradual re-uptake of the amine after the deenergization by FCCP or valinomycin plus K^+ , most likely evidences the activity of the above cited energy independent transport operating after the deenergization (Fig. 21).

Considering that serotonin can be metabolized by MAO with the production of aldehyde, we tested the effects of the inhibitors clorgyline and idazoxan.

Clorigyline is a propargylamine that inhibits the MAO activity as well as the serotonin uptake. Idazoxan, an inhibitor of I_2 imidazoline receptor, exhibits an inhibition of uptake very similar to that exerted by clorgyline.

The observation that both compounds decrease the energy dependent uptake of serotonin on the one hand suggests that the species accumulated is the aldehyde, but by considering that the transport has an electrophoretic behaviour, another proposal could be that the transporter is located in a domain belonging to MAO. In this regard the possible involvement of the I₂ receptor should be taken into account.

The fact that RBM exhibit a different behaviour respect the other types of mitochondria in MPT induction may be related to the different localization of MAOs in the outer membrane as previously reported by Wang and Edmondson (2011). This may provoke a different site of H_2O_2 production that in RBM could be away from the pore forming structures, therefore the critical SH groups should not be oxidized and the pore would remain closed.

Discussing the results obtained by this investigation I wish to emphasize that the initial aim of it, that is the study of monoamine transport and function in mitochondria has been shifted toward a different objective that is the individualization of an important different mechanism in RBM as respect the other mitochondria types.

At present the physiological meaning of this difference is unknown. A possible explanation is that H_2O_2 produced by MAOs is addressed towards other RBM targets. It is to emphasize that several typical inhibitors, in particular the antioxidants, fail to protect RBM against MPT induction by Ca²⁺, so it will be to revalue old theories suggesting that the pore opening does not require oxidative stress. Furthermore it is also to emphasize that the discovery of Src kinases and phosphatases together Tyr-phosphorylated proteins in RBM strongly supports the existence of a sophisticated regulatory mechanism of MPT induction in these mitochondria (see next chapter).

The effects on MPT amplification in RLM and the other mitochondria by monoamines are similar to those observed with other oxidants, suggesting that the mechanism is related to an oxidative stress.

Instead, an important difference has been observed at the level of the uptake mechanism. In fact, it has been evidenced that the initial uptake of serotonin seems to be a sum of a membrane binding and a very rapid energy independent accumulation of the amine. This transport could take place through the I₂ receptor as idazoxan partially inhibits it. However this proposal requires other confirms.

In conclusion serotonin, but also other monoamines, are transported by two types of mechanisms, an energy independent and one energy dependent. Experiments are in progress in our laboratory in order to determine the physiological role of monoamine transport. The different localization of MAOs in the different types of mitochondria could be related to the efficiency of the transport.

Phosphorylation/ dephosphorylation state in mitochondria

Protein phosphorylation is an important event implicated in a large number of cell processes, particularly enzymatic regulation and signal transduction pathways (Hunter 1995). Signal transduction mediated by protein phosphorylation is generally triggered by cell surface receptors with tyrosine kinase activity, and for this reason, the plasma membrane has been considered the main subcellular site of such activities (Schenk and Snaar-Jagalska 1999). Tyrosine phosphorylation however has also been identified in endoplasmic reticulum membranes and in the nuclear envelope (Brown and Cooper 1996).

Besides receptor tyrosine kinases, tyrosine kinases including by the Src family (Src, Fyn, Lyn, Fgr, etc.) has been identified, which may serve as control switches in a variety of signal transduction pathways regulating essential cell processes (Erpel and Courtneidge 1995). Tyrosine phosphorylation is a reversible process that depends on the activity of both tyrosine kinases and tyrosine phosphatases.

Inhibition of cellular tyrosine phosphatase activity likely causes increasing levels of tyrosine-phosphorylated proteins, which implies the existence of a dynamic relationship between tyrosine phosphorylation and dephosphorylation pathways. Conditions of oxidative stress may modify the activity of major signaling enzymes, including tyrosine phosphatases and kinases (Rahman et al. 1993).

It is known that mitochondria participate in various processes involving signal transduction pathways, including apoptosis (Skulachev 1999), oxidative stress and neoplastic proliferation (Crompton and Costi 1988; Skulachev 1999; Gottlieb 2000). In the last years many studies were focused on the phosphorylation activity at mitochondrial level, and Ser/Thr as well as Tyr-phosphorylation has been studied in depth (Piedimonte et al. 1986, 1988a, 1988b; Ferrari et al. 1990; Technikova-Dobrova et al. 1993; Papa et al. 1999; Salvi et al. 2002)

The discovery of several protein kinases localized within mitochondria opens new perspectives on the regulation of signals and the involvement of mitochondria on different aspects of cell life that require an integrated signal system mediated by factors entering and leaving from these organelles in according to the cell needs.

Emerging data indicate that the reversible phosphorylation, the major form of post-translational modification, is an important regulatory process of mitochondrial functions. The increasing number of mitochondrial kinases reported in literature, suggests that phosphorylation is emerging as a common step in the regulation of processes implemented by these organelles.

Studies concerning the mitochondrial functions regulated by protein kinases, and the localization in mitochondrial compartments of these enzymes show that different effects are elicited by different; for example protein kinase A antagonizes apoptosis (Huang et al. 2001), while protein kinase C has proapoptotic activity (Farrow et al. 2002), and the proliferation of many cell types are stimulated by protein kinase B/Akt (Lawlor and Alessi 2001).

Mitochondrial phoshorylation involves not only serine/threonine but also histidine and tyrosine residues, in particular the mitochondrial tyrosine phosphorylation is emerging as a central mechanism in the regulation of mitochondrial functions (Salvi et al. 2005; Pagliarini and Dixon 2006). In rat brain mitochondria several tyrosine phosphorylated proteins, such as ADP/ATP translocase (AdNT), exokinase, creatine kinase and ATP synthase, were discovered (Lewandrowski et al. 2008).

Results

Considering that RBM, at variance with RLM, contain tyrosine kinases (Salvi et al. 2002; Salvi et al. 2005; Arachiche et al. 2008) the involvement of these enzymes in the MPT process has been investigated. In this regard RBM were incubated in the presence of specific tyrosine kinase inhibitors such as genistein and PP2, and the results of Fig. 25 show no appreciable effects of these compounds on the Ca^{2+} - induced swelling.





In order to remove any doubt about the possible involvement of kinase in the MPT process, the inhibitors of tyrosine phosphatase were tested. Contrary to what expected the results obtained by incubating RBM with these inhibitors showed that pervanadate, a potent phosphatase inhibitor, strongly inhibits the swelling induced by calcium and "Inhibitor Tyr- Phosphatases Cocktail 2" delays the swelling. On the contrary the "Inhibitor Ser/Thr-Phosphatases Cocktail 3" does not alter the Ca²⁺-induced swelling (Fig. 26).





Incubation conditions as described in Material and Methods section. The medium was supplemented with 100 μ M Ca²⁺ except when otherwise indicated and when reported 10 μ I/mg prot Inhibitor Tyr-Phosphatases Cocktail 2, 10 μ I/mg prot prot Inhibitor Ser/Thr-Phosphatases Cocktail 3 and 1 mM pervanadate were present. Traces are representative of four experiments.

In order to discriminate the effect of the different components of the "Cocktail 2" we tested the action of single compounds constituting the cocktail such as molibdate and imidazole. Fig. 27 shows a slight protection on the Ca²⁺-induced swelling by imidazole and an inhibition of about 50% displayed by molibdate.



Fig. 27 Effect of tyrosine phosphatase inhibitors on MPT in RBM.

Incubation conditions as described in Material and Methods section. The medium was supplemented with 100 μ M Ca²⁺ except when otherwise indicated and when reported 500 μ M molibdate, and 3 mM imidazole were present. Traces are representative of four experiments.

In order to evaluate the effects of these inhibitors on the mitochondrial protein phosphorylation a Western Blotting analysis was performed.

The pattern of phosphorylation in the presence of these compounds in RBM is reported in Fig. 28.





Incubation conditions as described in Material and Methods section. The medium was supplemented with 100 μ M Ca²⁺ except when otherwise indicated and when reported 10 μ l/mg prot Inhibitor Tyr-Phosphatases Cocktail 2, 3 mM imidazole, 500 μ M molibdate and 1 mM pervanadate were present. Results are representative of five experiments.

The degree of phosphorylation in the presence of " Cocktail 2", imidazole and molibdate in RBM (with Ca²⁺) is higher than that found in control conditions. The effect is particularly evident on the protein bands of about 50 and 60 KDa in the case of "Cocktail 2" and imidazole, and on protein bands of 65 KDa in the case of molibdate. As expected pervanadate strongly increases the phosphorylation degree of several bands.

The experiments on RLM are in progress in order to evaluate the effects of these compounds in these type of mitochondria.

Discussion

The involvement of mitochondria in different aspects of cellular life is due to the existence of an integrated system of signals, since mitochondria are multifunctional organelles that participate in a variety of cellular processes such as energy production, proliferation, senescence and death. The discovery of several protein kinases and phosphatases localized inside the mitochondria opens new perspectives in the regulation of these signals. This third part of the thesis focused on protein tyrosine phosphorylation and dephosphorylation in mitochondria. In fact, analysis of protein phosphorylation in RBM revealed the presence of several phosphoproteins whose phosphorylation strongly increases upon treatment with pervanadate and Mn²⁺, indicating a mitochondrial tyrosine phosphorylation (Salvi et al. 2002). Three prominent bands, with apparent M.W. of 50, 60, and 75 kDa, were detected by anti-phosphotyrosine blotting. This phosphorylation vanish when mitochondria are treated with PP2, a Src kinase family inhibitor, suggesting the presence of members of this family in RBM. Several experiments showed that tyrosine-phosphorylated proteins are membrane-bound and that they are located on the inner surface of the outer membrane and/or the external surface of the inner membrane. Instead, Src tyrosine kinases are mainly located in the intermembrane. Rat brain mitochondria were also found to possess a marked level of tyrosine phosphatase activity, strongly inhibited by pervanadate, a well-known inhibitor of tyrosine phosphatases, but not by inhibitors of alkali or Ser/Thr phosphatases, and mainly take place in the intermembrane space and outer mitochondrial membrane. Using a combination of techniques in mitochondria, Salvi et al. (2004) identified the tyrosine phosphatase Shp-2 that plays a crucial role in a number of intracellular signalling cascades and it is probably involved in several diseases.

On the basis of these remarks and considering that liver mitochondria do not possess such kinases the experiments with RBM were conducted with the aim to assess the possible effects of kinase and phosphatase activity in these organelles, given their different behavior in the induction of MPT than RLM. We then evaluated the possible effect of kinases on MPT in brain mitochondria utilizing specific inhibitors of tyrosine kinases such as PP2 and genistein but the results did not show any effect by them on the opening of the PTP (Fig. 25). We then tested whether phosphatases were involved in the process because of their presence in the RBM (as described above). As shown in Fig. 26, pervanadate strongly inhibits the swelling induced by calcium, while the "Inhibitor Ser/ThrPhosphatases Cocktail 3" has no effect on the MPT in RBM. The "Inhibitor Tyr-Phosphatases Cocktail 2" shows an interesting behaviour, in fact in a first moment it exerts a strong protective effect but this decreases with time. Being "Cocktail 2" a mixture of compounds, we tested them separately in order to better clarify such effect. Imidazole protects only slightly the Ca²⁺-induced swelling whereas molibdate exhibits an inhibition of about 50% on the swelling (Fig. 27).

Analyzing the effects of these compounds on the phosphorylation pattern of mitochondrial proteins it is immediately clear that the pervanadate causes an increase in the degree of phosphorylation of several proteins, which correlates with its protective effect on the swelling induced by calcium. The degree of phosphorylation in the presence of the "Cocktail 2", imidazole and molibdate is greater than that observed in RBM incubated with Ca²⁺ alone and this effect is particularly evident on some protein bands.

In conclusion, the tyrosine phosphorylation pattern seems to be consistent with the hypothesis that an high phosphorylation degree could mantain the permeability transition pore in the closed conformation, while a low phosphorylation degree could permit the opening of pore and the consequent permeability transition.

These observations evidence for the first time a strong difference in the induction and regulation of MPT between RBM and the other types of mitochondria, thus demonstrating a tissue specificity for this process. In particular MPT in RLM, RKM, and RHM is induced by the interaction of Ca²⁺ with specific sites located on AdNT or phosphate transporters and by ROS produced, in our case, by monoamine oxidation. Thus, in the mitochondria MPT is regulated by the redox level of the organelles. In RBM the MPT induction is also provoked by the level of Ca²⁺ but the effect of ROS seems not to be a key mechanism for this process, or, at least they may have only a partial effect. Instead the main mechanism for the induction and regulation of MPT in RBM seems to be the tyrosine phosphorylation/dephosphorylation degree of some not yet well defined proteins by Src kinases and phosphatases.
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