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Tyramine and Monoamine Oxidase Inhibitors as Modulators of the Mitochondrial Membrane Permeability Transition

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Abstract. Incubation of rat liver mitochondria with 100–500 μM tyramine, a substrate for monoamine oxidases A and B (MAOs), in the presence of 30 μM Ca^{2+} induces matrix swelling, accompanied by collapse of membrane potential, efflux of endogenous Mg^{2+} and accumulated Ca^{2+} and oxidation of endogenous pyridine nucleotides. These effects are completely abolished in the presence of cyclosporin A, ADP, dithioerythritol and N-ethylmaleimide, thus confirming the induction of the mitochondrial membrane permeability transition (MPT). The observed partial protective effect exerted by catalase indicates the involvement of both MAO-derived hydrogen peroxide and aldehyde. Higher concentrations of tyramine (1–2 mM) are less effective or even completely ineffective. At these high concentrations tyramine has an inhibitory effect when the MPT is induced by 100 μM Ca^{2+}. The MAO inhibitors clorgyline (50 μM) and pargyline (500 μM) completely protect against MPT induction by 100 μM tyramine but also inhibit the phenomenon, although with different efficacy, when it is induced by 100 μM Ca^{2+} in the absence of tyramine. Taken together, our data suggest that tyramine, clorgyline and pargyline act as modulators of the MPT either through a direct inducing/protective effect or by controlling hydrogen peroxide and aldehyde generation.

Key words: Mitochondria — Permeability transition — Tyramine — Clorgyline — Pargyline — Monoamine oxidase — Hydrogen peroxide

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Introduction

The mitochondrial membrane permeability transition is a phenomenon characterized by large-amplitude mitochondrial swelling, the collapse of membrane potential ($\Delta\psi$), the oxidation of membrane thiols and endogenous pyridine nucleotides (PN), and the opening of a 3-nm pore, called the megachannel. The rapid change in permeability associated with the MPT causes uncoupling of oxidative phosphorylation and release of intramitochondrial ions and metabolic intermediates responsible for large-amplitude swelling. The presence of phosphate (Pi) and oxidants facilitates the onset of the MPT, whereas Mg^{2+}, ADP, polyamines, acidic matrix pH and high $\Delta\psi$ oppose it (for reviews on the MPT, *see*: Gunter & Pfeiffer, 1990; Zoratti & Szabò, 1995). The immunosuppressive cyclic endecapeptide cyclosporin A (CsA) specifically blocks onset of the MPT (Fournier, Ducet & Crevat, 1987; Crompton, Ellinger & Costi, 1988), thus indicating that the phenomenon involves a protein channel rather than a nonspecific perturbation of the lipid bilayer (Crompton, Costi & Hayat, 1987).

The complex relationships among biogenic amines, the MPT and apoptosis remain to be understood. Mitochondrial damage leading to apoptosis or necrosis can be triggered by the aldehydes and hydrogen peroxide produced during the oxidative deamination of biogenic amines by MAOs, a family of FAD-containing enzymes present on the outer membrane of mitochondria.

The involvement of MAO activity in the processes regulating cellular death is substained by the ability of clorgyline and pargyline, specific inhibitors of MAO A and B, respectively (Youdim & Finberg, 1991), to protect cultured human melanoma cells

from apoptosis induced by serum starvation (Malorni et al., 1998) as well from cytotoxicity induced by serotonin (Buckman, Sutphin & Mitrovic, 1993). Oxidative damage to rat brain mitochondrial DNA has been reported to occur upon the metabolism of tyramine by MAOs (Hauptmann et al., 1996) and it has been demonstrated that H_2O_2 generated by MAO activity enhances the release of Ca^{2+} and glutathione from mitochondria (Sandri, Panfili & Ernster, 1990). Moreover, a specific release of Ca^{2+} from mitochondria is known to be stimulated by reactive oxygen species (ROS), e.g., superoxide radicals, hydroxyl radicals and H_2O_2, and in its turn mitochondrial ROS production followed by enhanced Ca^{2+} cycling is well known to cause apoptosis and necrosis (Richter et al., 1995).

Generation of ROS by monoamines (MA), associated with the activity of glutathione oxidase and glutathione reductase, provokes pyridine nucleotides and protein thiol oxidation. In particular, it has been reported (Cohen & Kesler, 1999) that tyramine oxidation catalyzed by endogenous MAOs, in both brain and liver mitochondria, results in impaired respiratory activity, attributable to the formation of protein-glutathione mixed disulfide, which inhibits Complex I of the respiratory chain and other thiol-dependent enzymes of the inner membrane.

ROS generation has been suggested to increase the probability of induction of the MPT. It has also been proposed that pore formation and apoptosis are involved in the organization of a defence system that, by controlling ROS production, can eliminate those cells that become unwanted by the organism (Skulachev, 1999).

To assess the molecular mechanism by which the oxidative deamination of biogenic amines by MAO activity may control cellular death processes at the mitochondrial level, in the present paper we investigated the relationship between the oxidation of tyramine, a substrate of MAOs A and B, and induction of the MPT, using isolated rat liver mitochondria (RLM) as a model system. Data are discussed in the context of a hypothesis proposing double mechanisms of action of tyramine, as well as clorgyline and pargyline, on the MPT, in which these amines act by modulating H_2O_2 and aldehyde production or by direct interaction with the permeability transition pore.

Materials and Methods

Materials

Hepes, EGTA, rotenone, homovanillic acid, horseradish peroxidase, N-ethylmaleimide, dithioerythritol, hydroxyphenylacetaldehyde, pargyline, clorgyline, hydrogen peroxide and tyramine hydrochloride were purchased from Sigma-Aldrich. Catalase was from BDH. Cyclosporin A was kindly provided by Sandoz Pharma, Basel, Switzerland. All other reagents were of the highest quality available.

Mitochondrial Preparations

Fed female Wistar-derived rats (200–300 g) maintained on a standard diet were sacrificed by cervical dislocation after stunning. Liver mitochondria were prepared by homogenization, followed by conventional differential centrifugation, as previously indicated (Schneider & Hogeboom, 1950), in ice-cold medium containing 250 mM sucrose, 5 mM Hepes and 1 mM EGTA adjusted to pH 7.4 with HCl. After initial pelleting and washing steps, the mitochondria were washed in medium containing 250 mM sucrose and 5 mM Hepes, pH 7.4. The mitochondrial protein content was determined by the biuret method, with bovine serum albumin as a standard (Gornall, Bardawill & David, 1949). Mitochondria were stored on ice at a concentration of 40–60 mg of protein/ml and used within 4 hr of preparation.

Standard Incubation Procedures

Incubations were carried out at 20°C in a water-jacketed cell with 1 mg of mitochondrial protein/ml suspended in a standard medium used in previous permeability transition studies (Crompton, Costi & Hayat, 1987; Halestrap & Davidson, 1990; Beutner et al. 1996; Luo et al. 1998; Skulachev, 1999). The medium contained 200 mM sucrose, 10 mM Hepes (pH 7.4), 5 mM succinate, 1.25 μM rotenone, 30 μM calcium chloride and 1 mM Pi. Sodium salts were used. Rotenone was included in the medium in order to avoid the formation of oxalacetate, a strong competitive inhibitor of succinate dehydrogenase (Pardee & Potter, 1948) and to obtain optimal succinate oxidation. Other additions are indicated in the figure legends.

The experiments were carried out at 20°C in order to compare the results with those obtained in other previous papers on the MPT (Tassani et al., 1995; 1996). Whole rat liver mitochondria exhibit a reversible broad gel to liquid crystalline phase transition at 0°C (Blazyk & Steim, 1972), and at 20°C the membrane is in the sol form. Under MPT conditions the fluidity of the membrane is greatly increased with respect to control conditions (Ricchelli et al., 1999) and further increases with increasing temperatures. Therefore, the choice of 20°C was made with the aim of minimizing alteration of the membrane during the MPT due to excessive fluidity. Furthermore, it must be emphasized that at a higher temperatures, e.g., 30°C, the respiratory chain operates at high rate, producing anaerobiosis in the mitochondrial suspension within a few minutes, particularly under MPT conditions.

Determination of Mitochondrial Functions

Large-amplitude mitochondrial swelling was qualitatively measured by monitoring the decrease in light-scattering of a mitochondrial suspension at 540 nm in a 3-ml cuvette using a Kontron-Uvikon-922 spectrophotometer equipped with a magnetic stirrer and thermostatic control. Mitochondria were suspended as indicated above and, upon stabilization of the absorbance trace, swelling was assessed after additions of other compounds as described in the figure legends.

$\Delta\psi$ was measured by monitoring the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) across the mitochondrial membrane with a selective electrode prepared in our laboratory according to published procedures (Kamo et al., 1979; Affolter & Sigel, 1979) and an Ag/AgCl reference electrode. TPP^+ was included at a concentration of 2 μM in order to achieve high sensitivity in measurements and to avoid toxic effects on the proton ATPase and calcium flux (Jensen & Gunter, 1984; Karadyov et al.,

1986). The measurement was started by adding mitochondria to the incubation medium supplemented with other compounds, as indicated in the appropriate legends, after TPP^+ calibration. The membrane potential was calculated from the Nernst equation and corrected for nonspecific intramitochondrial binding of TPP^+ using the equation; $\Delta\psi = (\Delta\psi \text{ electrode} - 66.16 \text{ mV})/0.92$ (Jensen, Gunter & Gunter, 1986).

The mitochondrial matrix volume was calculated in parallel to $\Delta\psi$ from the distributions of [^{14}C] sucrose and ^3H_2O (Palmieri & Klingenberg, 1979) and was used to calculate the intramitochondrial concentration of TPP^+. Further details of these measurements are described in Toninello et al. (1992) and the bibliography therein.

The oxidation-reduction state of mitochondrial PN was monitored fluorometrically with excitation at 352 nm and emission at 464 nm in an Aminco Bowman spectrofluorometer (Moore et al., 1987). PN oxidation was followed as a decrease in fluorescence.

Ca^{2+} uptake and release and Mg^{2+} efflux were estimated by atomic absorption spectroscopy of the supernatant fraction (Crompton, Capano & Carafoli, 1976). Mitochondria (10 mg protein) were added to 10 ml of standard medium containing other compounds as indicated in the specific figure legends. The suspension was stirred continuously and, at intervals of time, 1-ml portions of the suspension were withdrawn and centrifuged for 2 min in an Eppendorf bench centrifuge (model 5415C) operating at 15,000 rev/min; this centrifugation was sufficient to sediment at least 98% of the mitochondria. The supernatant fluids were removed and their Ca^{2+} and Mg^{2+} contents determined by using a Perkin Elmer model 1100B spectrophotometer.

H_2O_2 production was determined fluorometrically (Matsumoto et al., 1982). Mitochondria at a concentration of 1 mg/ml were incubated at 20°C in standard medium containing horseradish peroxidase (9 U/ml), 0.9 mM homovanillic acid and tyramine and/or MAO inhibitors at indicated concentrations. At intervals of time, aliquots of the incubation mixture were withdrawn and combined with 0.1 M NaOH. The fluorescence was then evaluated using a Perkin-Elmer LS 50B spectrofluorometer with an excitation wavelength of 324 nm and emission at 426 nm. Fluorometric calibration curves were prepared under the same experimental conditions using serial concentrations of commercial H_2O_2 in the absence or presence of 1 mg/ml mitochondria. The presence of mitochondria did not modify the calibration curves for H_2O_2, thus indicating that the mitochondrial suspensions we used did not contain contaminating peroxisomal catalase that could interfere with the H_2O_2 assay.

Results

RLM incubated in standard medium containing 30 μM Ca^{2+} and 1 mM Pi do not exhibit any change in the apparent absorbance at 540 nm for at least 20 min of incubation. The addition of 100 μM tyramine, a substrate for both MAO A and MAO B, induces a decrease in absorbance, indicative of large amplitude matrix swelling (Fig. 1). The osmotic alteration induced by 100 μM tyramine in the presence of 30 μM Ca^{2+} is accompanied by the collapse of $\Delta\psi$ (Fig. 2), the efflux of endogenous Mg^{2+}, and the release of accumulated Ca^{2+} (Fig. 3*A* and *B*, respectively) as well as the oxidation of mitochondrial PN (Fig. 4), all phenomena indicative of MPT induction. No changes are observed when tyramine is added in the absence of 30 μM Ca^{2+}.

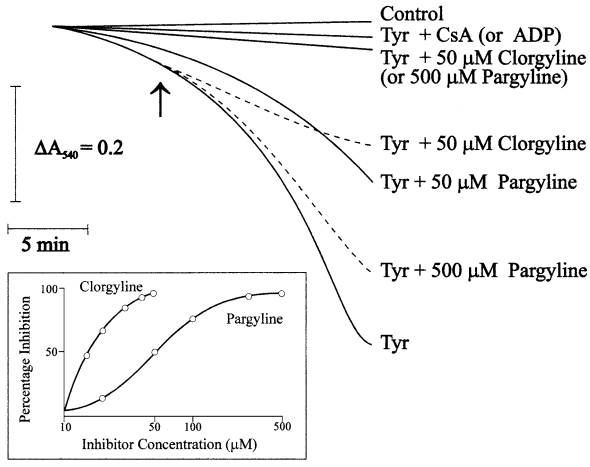

Fig. 1. Mitochondrial swelling induced by tyramine. Inhibition by CsA, ADP and MAO inhibitors. RLM were incubated in standard medium in the conditions indicated in Materials and Methods. When present: 100 μM tyramine (Tyr), 1 μM CsA, 1 mM ADP. Clorgyline and pargyline were added at the indicated concentrations. Dashed lines: clorgyline and pargyline added at arrow. A downward deflection indicates mitochondrial swelling. The assays were performed 9 times with comparable results. The inset shows the dose-dependent effect of the inhibitors on the MPT induced by 100 μM tyramine.

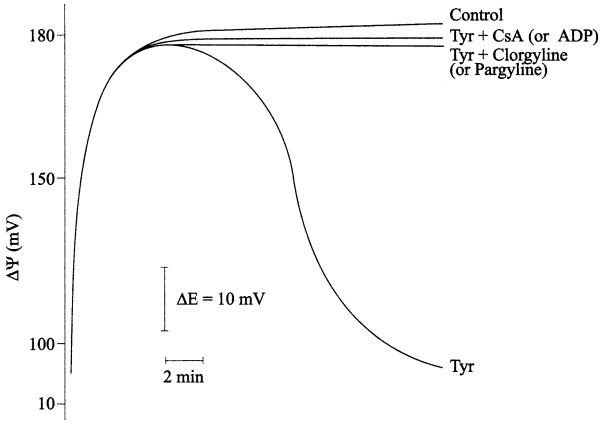

Fig. 2. Membrane potential collapse induced by tyramine and its inhibition by CsA, ADP and MAO inhibitors. Assay conditions and reagent concentrations were as described in Materials and Methods with 100 μM tyramine (Tyr), clorgyline at 50 μM and pargyline at 500 μM. Standard medium was supplemented with 2 μM TPP^+ for $\Delta\psi$ measurements. ΔE, electrode potential. Five additional experiments exhibited the same trend.

The effects of 30 μM Ca^{2+} and 100 μM tyramine are completely prevented by the immunosuppressant CsA (1 μM), a commonly used specific inhibitor of the MPT, by 1 mM ADP, a physiological inhibitor of the MPT (Figs. 1–4), and by the reducing agent DTE and the alkylating agent NEM, (at final concentrations of 5 mM and 10 μM, respectively) (Figs. 4, 5), indicating that tyramine acts on the permeability transition pore rather than through a nonspecific perturbation of the lipid bilayer (Crompton, Ellinger & Costi, 1988).

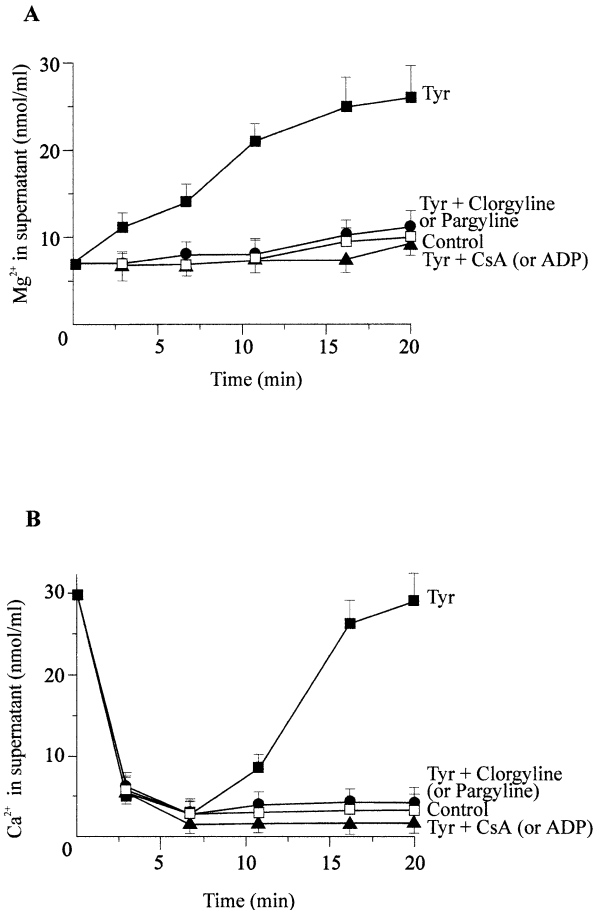

Fig. 3. Efflux of endogenous Mg^{2+} (*A*) and accumulated Ca^{2+} (*B*) induced by tyramine; prevention by CsA, ADP and MAO inhibitors. Assay conditions and reagent concentrations were as described in Materials and Methods with 100 μM tyramine (Tyr), clorgyline at 50 μM and pargyline at 500 μM. The initial endogenous concentrations of Mg^{2+} and Ca^{2+} were 27 and 10 nmol/mg protein, respectively. Reported are the mean values and SD from 6 experiments.

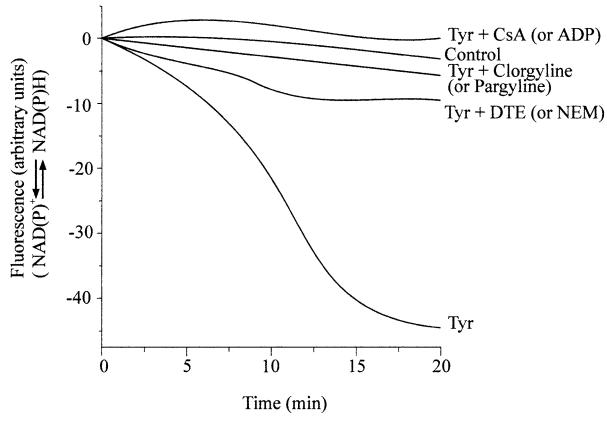

Fig. 4. Oxidation of endogenous pyridine nucleotides by tyramine; inhibition by CsA, ADP, DTE, NEM and MAO inhibitors. Assay conditions were as described in Materials and Methods; reagent concentrations were as follows: 100 μM tyramine (Tyr), 1 μM CsA, 1 mM ADP, 50 μM clorgyline, 500 μM pargyline, 5 mM DTE, 10 μM NEM. The experiment was started with the addition of tyramine. Shown is a typical experiment; 6 additional experiments gave comparable results.

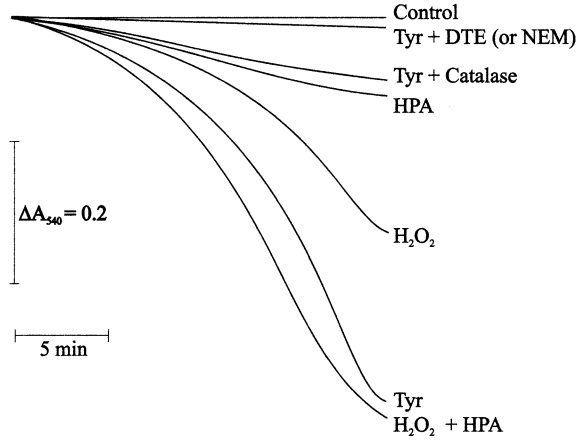

Fig. 5. Inhibition of tyramine-induced mitochondrial swelling by catalase, DTE and NEM, and a comparison of swelling induced by tyramine, hydrogen peroxide and hydroxyphenylacetaldehyde (HPA). Experimental conditions were as described in Materials and Methods. When present, 100 μM tyramine (Tyr), 5 mM DTE, 10 μM NEM, catalase (1000 U/mg prot), 100 μM H_2O_2, 100 μM HPA. The assays were performed 5 times with comparable results.

The addition of catalase (1000 U/mg mitochondrial protein) also inhibits the absorbance decrease induced by tyramine in the presence of Ca^{2+} (Fig. 5), thus suggesting that H_2O_2 produced during the oxidation of tyramine by MAOs is involved in MPT induction. The inhibition by catalase is, however, incomplete, indicating that hydroxyphenylacetaldehyde (HPA) produced during tyramine oxidation also contributes to the phenomenon. To confirm the involvement of H_2O_2 and aldehyde, we treated rat liver mitochondria with these substances (Fig. 5). H_2O_2 at a concentration of 100 μM is able to induce a change in the absorbance of mitochondrial suspensions, but to a lesser extent than 100 μM tyramine; HPA at the same concentration induces a very limited change in absorbance. However, addition of both 100 μM H_2O_2 and 100 μM HPA produces a synergistic effect in inducing MPT, which reaches the same extent as that observed upon addition of 100 μM tyramine.

The effects of 100 μM tyramine are also completely prevented in the presence of 50 μM clorgyline or 500 μM pargyline (Figs. 1–4), which are inhibitors of MAO A and MAO B, respectively (Youdim & Finberg, 1991). The effect of the inhibitors is dose-dependent and great differences are observed between them. As reported in the inset of Fig. 1, while clorgyline at 50 μM inhibits mitochondrial swelling by about 95%, pargyline at the same concentration exhibits an inhibitory effect of about 50%. Clorgyline and pargyline do not exert any effect on Ca^{2+} or Pi transport. Another inhibitor of MAO B, deprenyl,

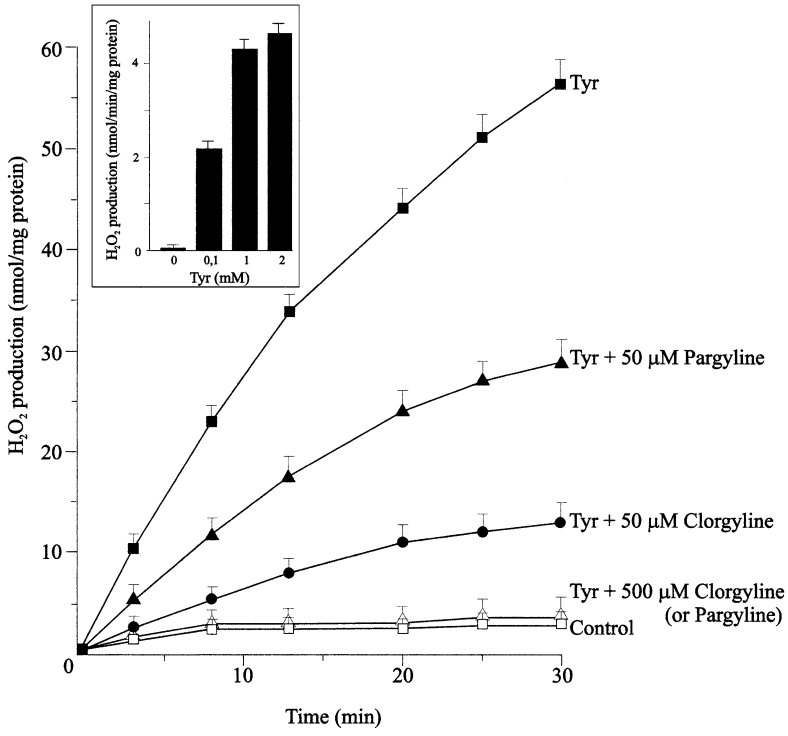

Fig. 6. Hydrogen peroxide production by rat liver mitochondria incubated in the presence of tyramine and the effect of MAO inhibitors. Assays were carried out as described in Materials and Methods, in the absence or presence of 100 μM tyramine (Tyr) and clorgyline and pargyline at the indicated concentrations. The inset reports the dose-response histograms of the initial rates of hydrogen peroxide production. Reported are the mean values and SD from 5 experiments.

shows similar results as those of pargyline (*results not reported*). The inhibitors are also able to inhibit MPT with different efficacy when they are added after the MPT is triggered (Fig. 1).

The results presented in Fig. 6 show the time course of mitochondrial H_2O_2 production due to the oxidation of 100 μM tyramine. It must be pointed out that the reduced amounts of H_2O_2 formation observed in these assays compared to values normally reported in the literature reflect the fact that the tyramine concentration employed lies slightly below the K_m and well below conditions for saturating MAO (Szutowicz, Kobes & Orsulak, 1984).

The inhibitory effects of clorgyline and pargyline on mitochondrial functions do not parallel their effects on amine oxidase activity. In fact, as reported in Fig. 6, while both inhibitors completely inhibit the MPT when added at a concentration of 500 μM, they exhibit different effects on the MPT and on H_2O_2 production when present at 50 μM: clorgyline completely blocks the MPT (Fig. 1) and inhibits H_2O_2 production from tyramine oxidation by 80%; on the other hand, 50 μM pargyline inhibits both H_2O_2 production and the MPT by 50% (inset in Fig. 1). These data indicate that the effect of pargyline on the MPT reflects the effect of the inhibitor on H_2O_2 and aldehyde generation. The observed ability of clorgyline to inhibit the MPT could be due to a mechanism that does not involve only the inhibition of tyramine oxidation. To test this possibility we evaluated the effect of MAO inhibitors on mitochondrial swelling induced by a high Ca^{2+} concentration in the absence of tyramine (Fig. 7). We observed that both clorgyline (50 μM) and pargyline (500 μM) inhibit the swelling induced by 100 μM Ca^{2+}, although with different efficacy. The same type of inhibition was observed also when the inhibitors were added after the phenomenon had been triggered. Furthermore, the same inhibitory effect by clorgyline or pargyline was also seen when the MPT was induced by *tert*-butylhydroperoxide or menadione (*results not reported*). These results clearly demonstrate that, at least in this type of experiment, clorgyline acts by a mechanism not linked to the MAO-dependent oxidation of tyramine.

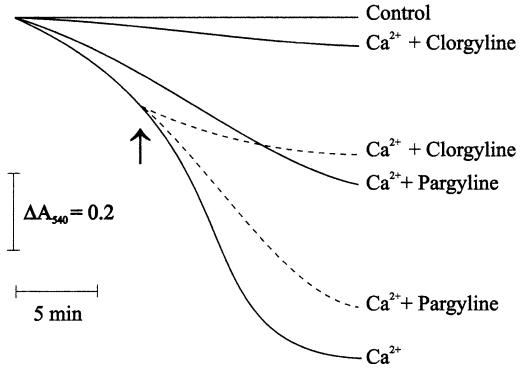

Fig. 7. Inhibition of Ca^{2+}-induced mitochondrial swelling by clorgyline or pargyline. Assay conditions were as described in Materials and Methods, with clorgyline and pargyline at 50 μM and 500 μM, respectively. Dashed line refers to clorgyline and pargyline added at the arrow. Ca^{2+} indicates that standard medium was supplemented with 70 μM Ca^{2+} in order to obtain a final cation concentration of 100 μM. The assays were performed 4 times with comparable results.

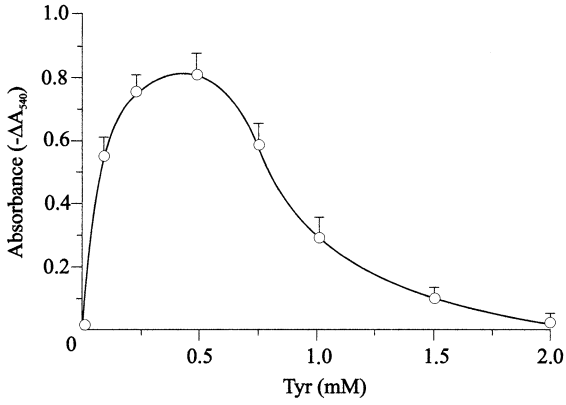

Fig. 8. Dose-dependent effect of tyramine on the induction of mitochondrial swelling. RLM were incubated in standard medium under the conditions indicated in Materials and Methods. Tyramine (Tyr) was present at the concentrations indicated by the points in the diagram. Reported are the mean values and SD from 5 experiments. Mitochondrial swelling was considered as the maximal extent of ΔA, which occurred after 20 min of incubation at each tyramine concentration.

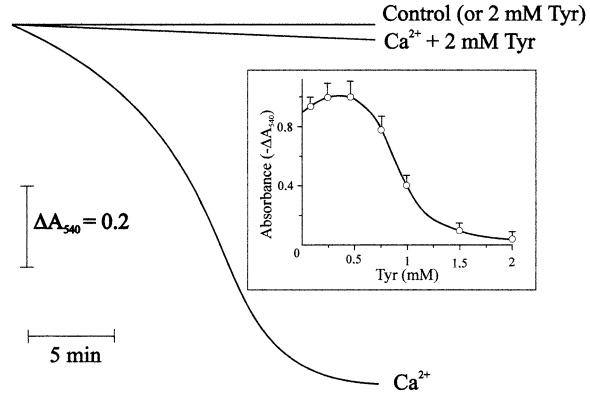

Fig. 9. Inhibition of Ca^{2+}-induced mitochondrial swelling by tyramine. Assay conditions were as described in Materials and Methods. Ca^{2+} indicates that standard medium was supplemented with 70 μM Ca^{2+} in order to obtain a final cation concentration of 100 μM. When present: 2 mM tyramine (Tyr). The assays were performed 5 times with comparable results. The insert reports a dose-response curve carried out in the presence of 100 μM Ca^{2+} and the indicated concentrations of tyramine. Reported are mean values and SD of 4 experiments.

The dose effect of tyramine on MPT was also investigated. As reported in Fig. 8, tyramine exhibits a maximal effect on mitochondrial swelling at a concentration of 500 μM and gradually loses its activity at higher concentrations, being ineffective at a concentration of 2 mM. We observed some oscillation at these two concentrations, possibly due to time-dependent variations in the status of the mitochondrial membranes. On the contrary, the effect of tyramine on the rate of H_2O_2 generation was dose-dependent and a plateau value was reached at a concentration between 1 and 2 mM (*see* inset in Fig. 6). These data indicate that tyramine acts as an inhibitor of the MPT when present at high concentrations, probably through mechanisms independent of H_2O_2 production. To test this hypothesis we evaluated the effect of tyramine on mitochondrial swelling induced by high Ca^{2+} concentrations. As reported in Fig. 9, tyramine inhibits mitochondrial swelling induced by 100 μM Ca^{2+}s; this effect is dose-dependent, with a complete blockage obtained at a concentration of 2 mM. At this concentration tyramine also completely blocks all the other events connected with mitochondrial swelling induced by Ca^{2+}, e.g., $\Delta\psi$ collapse, cation efflux, and oxidation of PN (*data not* shown).

All the above-reported results are reproduced when the standard sucrose medium is substituted with a saline medium.

Discussion

Mitochondria have recently been shown to play a central role in programmed cell death. Mitochondrial accumulation of ROS has been implicated in the induction of apoptosis upon treatment of a variety of agents. This phenomenon is, at least in part, related to ROS-induced mitochondrial Ca^{2+} cycling (Richter et al., 1995) and the induction of the MPT, as evidenced by the fact that cell death is effectively prevented by ruthenium red, a specific inhibitor of the mitochondrial Ca^{2+} uptake pathway (Kruman & Mattson, 1999).

The results reported here show that in the presence of 30 μM Ca^{2+}, tyramine at appropriate concentrations (100–500 μM) is able to trigger the MPT. In fact, it induces large-amplitude swelling (Fig. 1), the collapse of $\Delta\psi$ (Fig. 2), the efflux of endogenous Mg^{2+} and accumulated Ca^{2+} (Figs. 3*A* and *B*, respectively) and the oxidation of endogenous PN (Fig. 4), which are all prevented by typical MPT inhibitors such as CsA (Figs. 1–4), ADP (Figs. 1–4), and reducing and alkylating agents (DTE and NEM, respectively) (Figs. 4, 5).

Both of the products of MAO-dependent oxidation of tyramine, i.e., H_2O_2 and HPA, are involved in the stimulating effect of tyramine on the MPT. In fact, catalase incompletely protects mitochondria from the swelling provoked by tyramine (Fig. 5). Furthermore, direct addition of H_2O_2 or HPA to RLM is able to induce the MPT, with H_2O_2 more efficient than HPA. However, when H_2O_2 and HPA are added simultaneously, an apparent synergism in MPT induction is observed, as the addition of both molecules induces an osmotic alteration greater than that corresponding to the sum of each of their effects (Fig. 5). In this context, it should be pointed out that cultured cells are damaged both by H_2O_2 and aldehyde generated by the oxidation of amine by bovine serum amine oxidases (Averill-Bates et al., 1993).

The molecular composition of the permeability transition pore remains uncertain. It has been pro-

posed that the pore may be composed in part of the adenine nucleotide translocase (AdnT) (Halestrap & Davidson, 1990), as well as other proteins including cyclophilin (a CsA-binding protein in the matrix having peptidyl prolyl cis-trans isomerase activity), creatine kinase, porin and hexokinase (all present in the outer compartment) (Halestrap & Davidson, 1990; Zoratti & Szabò, 1995; Beutner et al., 1996; 1998). Other findings suggest that the pore comprises or is in close contact with Complex I proteins (Fontaine et al., 1998). A sequential model has been suggested for the Ca^{2+} and cyclophilin-dependent transformation of AdnT to the nonspecific CsA-sensitive transition pore (Halestrap & Davidson, 1990; Griffiths & Halestrap, 1991, and bibliography therein). In our specific case, in the presence of 30 μM Ca^{2+} (as observable in all the control experiments shown in Figs. 1–5), the pore remains closed in the absence of a second agent such as tyramine, whose oxidation to H_2O_2 and HPA triggers the MPT (Figs. 1–5). In contrast, a Ca^{2+} concentration of 100 μM induces the MPT in the absence of other agents (Fig. 9). In both cases, after the binding of a sufficient amount of Ca^{2+}, interaction of the pore-forming complex with cyclophilin requires the presence of Pi under these particular conditions.

Although oxidative stress has long been known to increase the probability of pore opening, the precise mechanism underlying its effect remains obscure. Results of previous studies indicate that two sites contribute to modulation of the MPT by oxidants (Bernardi, 1996). The results reported in Figs. 4 and 5 indicate that MPT induction by tyramine-derived H_2O_2, involving the oxidation of PN, and sensitive to NEM and DTE, most likely takes place by affecting both these sites. Moreover, an interaction of HPA with thiol and/or amino membrane groups is also possible. It must also be emphasized that aldehydes can be considered sources of ROS themselves (Kowaltowski et al., 1996).

In addition to observing MPT induction by tyramine, which is a substrate of both MAO A and MAO B, we also documented the phenomenon using 100 μM octopamine (specific for MAO A) and with 100 μM benzylamine (specific for MAO B) (*results not reported*). This finding demonstrates the involvement of both MAOs in the phenomenon.

While tyramine is able to induce the permeability transition at a concentration of 100 μM, corresponding to the amount normally present in the cytosol (Wagner, Claverie & Danzin, 1984), higher concentrations (1–2 mM) are partially or completely ineffective (*see* Figs. 1 and 8) or even inhibitory (Fig. 9). As the generation of H_2O_2 is dependent on the tyramine dose (inset of Fig. 6), the most likely explanation for the dose effect of tyramine on the MPT is that this amine begins to exhibit a phenomenon of protection *per se* at high concentrations; in other words, the tendency toward pore opening triggered by its oxidation is counteracted or abolished by the intact molecules not yet oxidized by MAOs. This proposal is strongly supported by the results reported in Fig. 9, which demonstrate that 2 mM tyramine is able to completely abolish mitochondrial swelling induced by the presence of excess Ca^{2+}. Such a direct effect of tyramine is in accordance with the general ability of a variety of molecules having one or more charged amino groups to protect against the MPT (i.e., polyamines, diamines, ruthenium red, carnitine, tetracaine, etc.) (Zoratti & Szabò, 1995).

When evaluated in the light of the observations of Cohen and Kesler (1999), the effect of tyramine on MPT reveals a more severe consequence on the interaction of MA with mitochondrial membranes. At a 500 μM concentration, besides inducing the maximum extent of the MPT (*see* Fig. 8), tyramine is also able to impair respiratory chain activity at the Complex I level (Cohen & Kesler, 1999). Indeed, by taking into account that this impairment of the electron flux is obtained in the presence of Mg^{2+}, a well-known inhibitor of the MPT, it is realistic to suggest that a higher tyramine concentration (2 mM), which also inhibits MPT (Fig. 9), would cause an even more accentuated damage to Complex I.

The involvement of Complex I in MPT induction has also been reported in rat skeletal muscle mitochondria (Fontaine et al., 1998). These authors demonstrate an inducing effect on the phenomenon, by increased electron flow through Complex I, which displaces ubiquinone from a regulatory binding site.

Taking into account the inhibitory effect exhibited by tyramine on Complex I of brain and liver mitochondria (Cohen & Kesler, 1999), comparisons with the results of Fontaine et al. (1998) suggest inhibition by tyramine on the MPT in muscle mitochondria at a concentration of 500 μM, which, for example, in our conditions with liver mitochondria, has an inducing effect (*see* Fig. 8).

However, the different experimental conditions of these studies (type of mitochondria, respiratory substrates, Pi concentrations) hinder these comparisons, which may indicate a tissue-specific effect of MA. Other studies in more appropriate conditions are required to confirm this hypothesis.

The induction of MPT may also be obtained by a pathway that differs from oxidation of the amine group by MAOs. The phenomenon has also been observed in brain and liver mitochondria when the catechol ring of dopamine is oxidized to quinone by tyrosinase (Berman & Hastings, 1999).

Similar to polyamines, MA are able to interact with a large number of K^+-channels found in a variety of cell membranes (*see* for example Liepins, LeFever & Truitt, 1989; Robinson et al., 1989; Schotland et al., 1995; Pearson & Nichols, 1998; Banchelli et al. 2000). Our finding that 2 mM tyramine

inhibits induction of the permeability transition (Fig. 9) could be explained just as an almost general inhibitory effect of MA on most K^+-channels.

The induction of MPT by tyramine is also inhibited by clorgyline and pargyline. The effect of 50 μM pargilyne on MPT induction (inset in Fig. 1) is in complete agreement with its effect on H_2O_2 formation (Fig. 6), that is, inhibition by about 50% of both the phenomena. Clorgyline at 50 μM completely inhibits the MPT (Fig. 1) and inhibits H_2O_2 by about 80% (Fig. 6). The different efficacies exhibited by the MAO inhibitors on MPT induction raise a question regarding their mechanism. It seems that the effect of 50 μM clorgyline is not solely due to inhibition of H_2O_2; another mechanism must also be involved. As reported in Fig. 7, the MPT induced by 100 μM Ca^{2+} in the absence of MAO substrate oxidation is partially inhibited to different extents by 50 μM clorgyline or 500 μM pargyline. One explanation for these results is that clorgyline and, with a lesser affinity, pargyline, which are propargylamines, bind to a critical site on the pore-forming protein and thereby exert their inhibitory effect.

In conclusion, clorgyline and pargyline inhibit MPT via 2 mechanisms, by inhibition of MAO activity and by their direct interaction with the mitochondrial membrane. This latter mechanism becomes evident for both the inhibitors when the phenomenon is induced by 100 μM Ca^{2+} in the absence of tyramine. The possible direct structural effect is further strengthened by the inhibition observed when the phenomenon is already triggered by tyramine (Fig. 1), 100 μM Ca^{2+} (Fig. 9), or *tert*-butylhydroperoxide (*result not reported*).

When considered in the context of the entire cell, the results of the present study, when considered with those of other authors (*see above*), lead to the proposal that, through their effects on MPT and respiratory chain activity, MAO substrates and fluctuations in their concentrations play a critical role in controlling not only apoptosis but also necrosis (Susin, Zamzami & Kroemer, 1998).

The ability of clorgyline and pargyline to inhibit apoptosis induced by serum starvation in a melanoma cell line (M14) (Malorni et al, 1998) or by the ROS produced by the oxidation of MA in neuron cultures (Buckman, Sutphin & Mitrovic, 1993) supports this proposal.

It has been reported very recently that (-)deprenyl is able to prevent apoptosis in dopaminergic neuroblastoma SH-SY5Y cells induced by an endogenous toxin, N-methyl (R) salsolinol, whereas clorgyline and pargyline are ineffective (Naoi et al., 2000). (-)Deprenyl prevent apoptosis through suppression of mitochondrial $\Delta\psi$ collapse. As both MPT induction and inhibition are stereochemical in nature (Naoi & Maruyama, 2001), this fact could explain both the very low concentrations necessary and the ineffectiveness of clorgyline and pargyline in inhibiting apoptosis. However, these observations do support the above-mentioned structural effect in MPT inhibition by MAO inhibitors.

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