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Research Article

Protective effect of *N*-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine (PF9601N) on mitochondrial permeability transition

V. Battaglia [a], E. Sanz [b], M. Salvi [a], M. Unzeta [b] and A. Toninello [a, *]

[a] Dipartimento di Chimica Biologica, Università di Padova, and Istituto di Neuroscienze del C.N.R., Unità per lo studio delle Biomembrane, Viale G. Colombo 3, 35121 Padova (Italy), Fax: +39 0498276133, e-mail: antonio.toninello@unipd.it
[b] Institut de Neurociences, Fac. de Medicina, 08193 Bellaterra, Barcelona (Spain)

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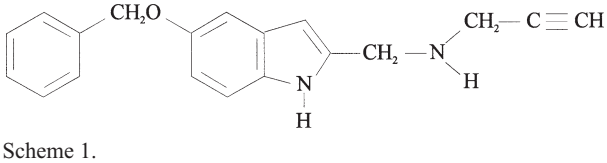
Abstract. PF9601N, *N*-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine, an monoamine oxidase (MAO) B inhibitor, has shown neuroprotective properties against dopaminergic toxins. To elucidate the mechanisms involved in this protection, the effect of PF9601N on mitochondria was assessed. PF9601N prevents mitochondrial swelling, drop in the electrical potential and oxidation of sulfhydryl groups, glutathione and pyridine nucleotides induced by Ca^{2+}. These observations demonstrate the protective effect of PF9601N on the induction of mitochondrial permeability transition. This protection is due to the interaction of the secondary protonated amino group in the molecule with pore-forming structures and to its antioxidant property, rather than to inhibition of MAO B activity. PF9601N also prevents the release of cytochrome c from mitochondria, suggesting its potential inhibitory effect on mitochondria-mediated apoptosis. The low IC_{50} value for this inhibition, in comparison with deprenyl, make it a more efficient compound than propargylamines and other amines in protecting the bioenergetic functions of mitochondria.

Keywords. Mitochondria, permeability transition, indolylalkylamines, calcium, monoamine oxidase inhibitors, reactive oxygen species.

Introduction

The compound *N*-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine (PF9601N) (scheme 1) is a new molecule, which turns out to be a monoamine oxidase (MAO) B inhibitor, more potent and selective than L-deprenyl (selegiline), used together with L-Dopa in treating Parkinson's disease. The absence of an amphetamine moiety in the chemical structure of PF9601N also confirms this new molecule as very useful in treating neurodegenerative diseases, since the side effects of L-deprenyl are avoided [1]. PF9601N has a neuroprotective effect *in vivo* in several experimental models using various dopaminergic toxins [2, 3]. It also enhances the duration of L-Dopa-induced contralateral turning in 6-hydroxydopamine lesioned rats [4] and has a protective effect on dopamine-lesioned PC12 cultured cells [5]. In addition, dopamine itself may contribute to cell death through its metabolism by MAO, which yields hydrogen peroxide as final catalytic product. In this context, the neuroprotection observed in PF9601N



Scheme 1.

* Corresponding author.

may be explained in terms of both inhibitory potency as an MAO B inhibitor, and/or the antioxidant properties of the 5-benzyloxy-indol-alkylamine derivatives previously observed *in vitro* [6]. Mitochondria represent the major source of the production of intracellular reactive oxygen species (ROS). Even in resting conditions, the reaction of oxygen with iron-sulfur centers in complex I, with partially reduced ubiquinone, and cytochrome b in complex III, generates superoxide ion and subsequently hydrogen peroxide and other ROS [7].

At supraphysiological Ca^{2+} concentrations, the generation of ROS by mitochondria induces the mitochondrial permeability transition (MPT) [8, 9], a phenomenon closely associated with programmed cell death involved in several diseases (for a review, see [10]). MPT is a physiopathological process which can initiate pathways to cell death by causing energy collapse, ATP depletion, promoting the release of cytochrome c (cyt c) and apoptosis inducing factor (AIF), and precipitating apoptotic cell death [11]. In this context, inhibition of the opening of the mitochondrial pore would be a good therapeutic target for use in treating several diseases.

In this regard, the aim of this work was to study the effect of PF9601N on MPT induced by Ca^{2+} and phosphate (P_i) in rat liver and brain mitochondria. The effect of PF9601N on oxygen uptake stimulated by Ca^{2+} and P_i and the level of reduced thiol groups, glutathione and pyridine nucleotides were also assessed in both types of mitochondria. Lastly, the inhibitory effect of PF9601N on MPT induction and subsequent prevention of the possible triggering of the pro-apoptotic pathway were evaluated by detecting cyt c release.

Materials and methods

Mitochondrial isolation and standard incubation procedures. Male Albino Wistar rats (200–250 g), kept in controlled conditions of temperature and humidity, were sacrificed by cervical dislocation. Liver mitochondria (RLM) were isolated by conventional differential centrifugation in an ice-cold buffer containing 250 mM sucrose, 5 mM HEPES (pH 7.4) and 1 mM EGTA [12]; EGTA was omitted from the final washing solution.

Brain mitochondria (RBM) were isolated and purified by the Ficoll gradient method, according to Nicholls [13], with some modifications [14]. Essentially, cerebral cortex was homogenized in isolation medium (320 mM sucrose, 5 mM HEPES, 0.5 mM EDTA, pH 7.4) plus 0.3% bovine serum albumin (BSA) and subjected to centrifugation (900 *g*) for 5 min. The supernatant was centrifuged at 17 000 *g* for 10 min to precipitate crude mitochondrial pellets. The pellets were resuspended in isolation medium plus 1 mM ATP and layered on top of a discontinuous gradient of Ficoll diluted in isolation medium, composed of 2-ml layers of 16% (w/v), 14% and 12% Ficoll, and a 3-ml layer of 7% Ficoll. After centrifugation for 30 min at 75 000 *g*, mitochondrial pellets were suspended in isolation medium and centrifuged again for 10 min at 800 *g*. The resulting pellets were suspended in isolation medium without EDTA.

The integrity of mitochondrial preparations was evaluated by measuring membrane potential ($\Delta\Psi$) values, ≥180 mV in RLM and 155 mV in RBM, and respiratory control ratios (RCR), ≥6 in RLM and ≥4 in RBM.

Protein content was measured by the biuret method with BSA as standard [15]. The mitochondrial matrix volume was calculated in parallel with the $\Delta\Psi$ from the distribution of [^{14}C]sucrose and ^3H_2O [16], and was used to calculate the intramitochondrial concentration of tetraphenylphosphonium (TPP^+).

Mitochondria (1 mg protein/ml) were incubated in a water-jacketed cell at 20 °C. The standard medium contained 250 mM sucrose, 10 mM HEPES (pH 7.4), 5 mM succinate, 1.25 µM rotenone and 1 mM P_i. Variations and/or other additions are given with each experiment.

Experiments were carried at 20 °C because the respiratory chain of isolated mitochondria at higher temperatures, *e.g.* 37 °C, operates at a high rate. As the amount of O_2 dissolved in *in vitro* conditions in the suspension medium is limited, anaerobiosis should be reached within a few minutes, resulting in the bioenergetic collapse of mitochondria. It should be noted that, at 20 °C, the membrane is in the sol form, as mitochondria exhibit a reversible broad gel to liquid crystal line phase transition at 0 °C [17], thus excluding the possibility of any great change in the crystalline phase of the membrane between 20 ° and 37 °C.

Determination of mitochondrial functions. $\Delta\Psi$ was calculated on the basis of the distribution of the lipid-soluble cation TPP^+ across the inner membrane, measured using a TPP^+-selective electrode [18] and an Ag/AgCl reference electrode. $\Delta\Psi$ was calculated from the Nernst equation and corrected for nonspecific intramitochondrial binding of TPP^+ by the equation: $\Delta\Psi = (\Delta\Psi_{electrode} - 66.16\ mV)/0.92$ [19].

Mitochondrial swelling was qualitatively determined by measuring the apparent absorbance decrease of mitochondrial suspensions at 540 nm in a Kontron Uvikon model 922 spectrophotometer equipped with thermostatic control. Each assay was started by the addition of mitochondria.

Mitochondrial oxygen uptake was monitored by a Clark electrode. Mitochondria were placed in a 1-ml sealed vessel under continuous magnetic stirring of the suspension. Oxygen concentration was taken as 480 nmol/ml at 20 °C. This measurement allowed calculation of the rates of states 3 (ADP-stimulated) and 4 (absence of ADP) respiration, and the RCR.

The redox state of endogenous pyridine nucleotides was followed fluorometrically in an Aminco-Bowman 4-8202 spectrofluorometer, with excitation at 354 nm and emission at 462 nm. The protein sulfhydryl oxidation assay was performed using 5,5′-dithiobis(2-nitrobenzoic acid) at 412 nm in a Kontron Uvikon Model 922 spectrophotometer, according to Santos et al. [20]. The redox level of glutathione was monitored as described in Tietze [21].

Cyt c release was determined in the incubation supernatant as reported by Salvi et al. [22]. Aliquots of supernatant fraction were concentrated by ultrafiltration through Centrikon 10 membranes (Amicon), subjected to 15% SDS-PAGE, and analyzed by Western blotting with mouse anti-cyt c antibody.

Results

It is well known and widely reported in the literature that, when mitochondria are treated with supraphysiological Ca^{2+} concentrations in the presence of P_i, they undergo the phenomenon of MPT, with opening of the transition pore [8].

This phenomenon is evidenced by extensive swelling of mitochondria in suspension, revealed by an apparent absorbance decrease at 540 nm of about 1 unit for RLM, and of lesser extent, about 0.2 units, for the RBM, when the organelles are incubated in standard medium with 50 μM Ca^{2+} and 1 mM P_i (Figs. 1a, 2a). In these experimental conditions, MPT is also characterized by complete

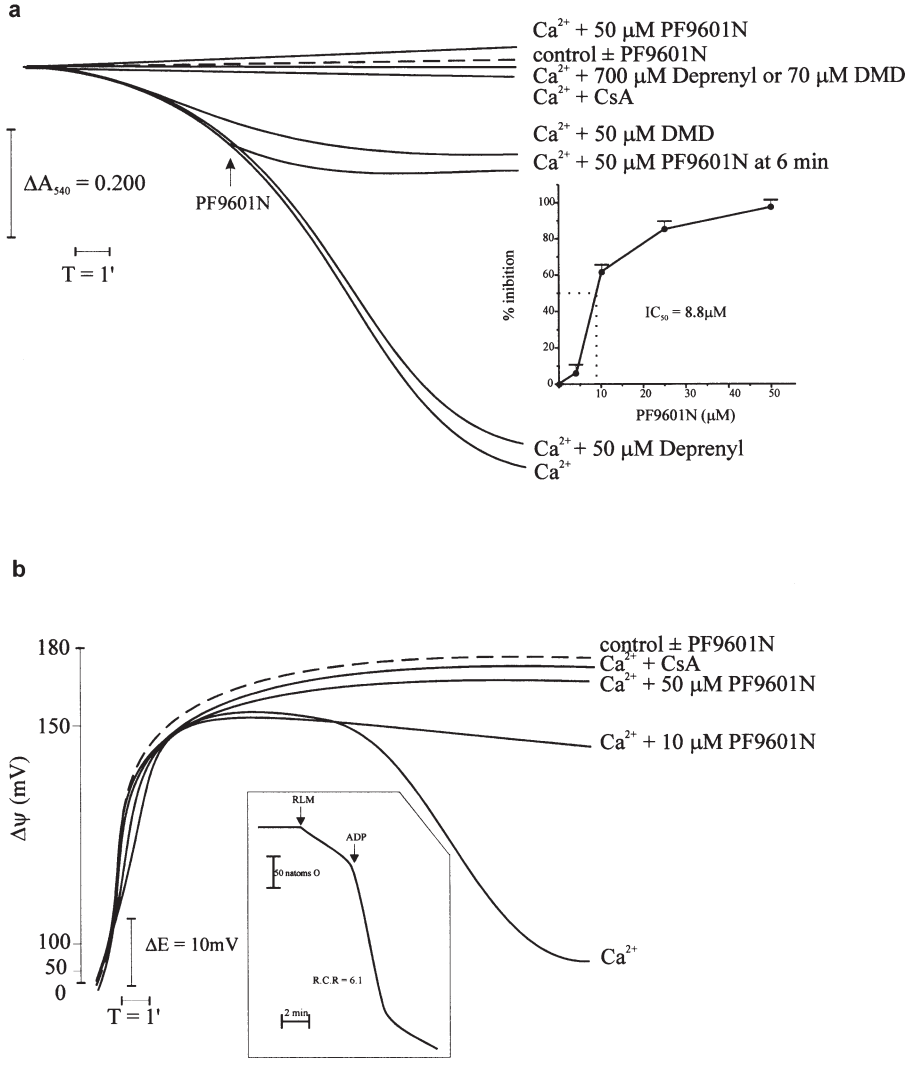


Figure 1. Swelling of RLM (*a*) and collapse of $\Delta\Psi$ (*b*), induced by Ca^{2+}, and inhibitory effect by PF9601N. RLM were incubated in standard medium in conditions indicated in Materials and methods. Control trace, also for subsequent experiments (dashed lines in all figures) refers to mitochondria incubated in standard medium in absence of Ca^{2+}. Where indicated, 50 μM Ca^{2+}, 1 μM CsA and 50 and 10 μM PF9601N were present. Deprenyl and desmethyldeprenyl (DMD) were present at indicated concentrations. (*a*) Downward deflection: mitochondrial swelling; arrow indicates the addition of PF9601N. Inset: calculation of IC_{50} value for PF9601N evaluated as percentages of ΔA extent after 15 min, with respect to that of control. (*b*) TPP^+ (1 μM) was added for $\Delta\Psi$ measurements. ΔE: electrode potential. Inset: RCR measurement. RLM incubated in standard medium, 250 μM ADP was added. Data are representative of six similar experiments.

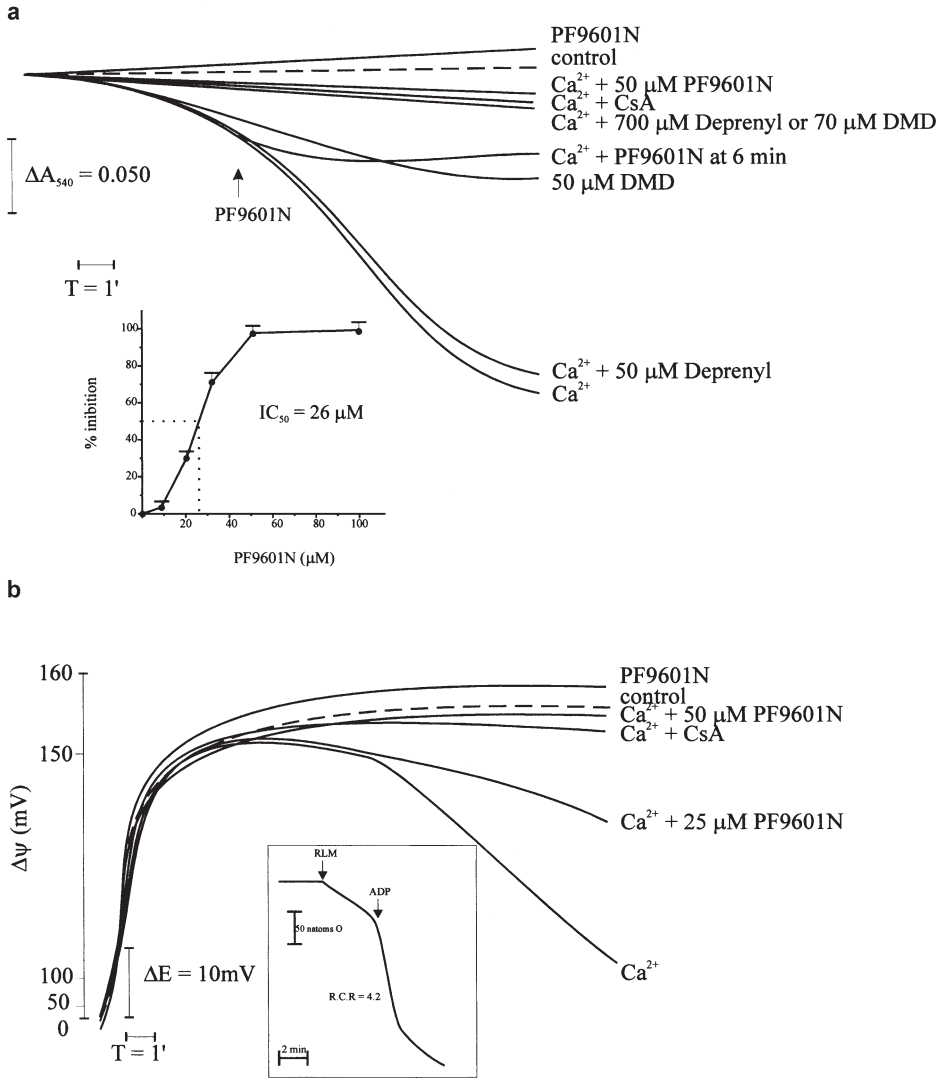


Figure 2. Swelling of RBM (*a*) and collapse of $\Delta\Psi$ (*b*), induced by Ca^{2+}, and inhibitory effect by PF9601N. Incubation conditions, reagent concentration and experimental details as in Fig. 1, PF9601N was present 50 and 25 μM as indicated. Inset *a*: calculation of IC_{50} value for PF9601N in RBM evaluated as in Fig. 1a. Inset *b*: RCR measurement as in Fig. 1*b*. Data are representative of four similar experiments.

collapse of the electrochemical gradient, shown here by the drop in $\Delta\Psi$ (Figs. 1b, 2b). The incubation of RLM and RBM with Ca^{2+} and P_i also induces stimulation of oxygen uptake (Figs. 3a, 3b, respectively) and a decrease, after 15 min of incubation, of about 40% and 25% of reduced thiol groups and glutathione, respectively, in RLM (Figs. 4a, 4b) and about 20% and 30%, in RBM (Figs. 5a, 5b). Indeed, under these experimental conditions, extensive oxidation of the $NAD^+/NADP^+$ pool takes place in both types of mitochondria, as observed by the great decrease in fluorescence at 366/450 nm (Figs. 6a, 6b).

When PF9601N is present in the incubation medium at 50 μM, it causes complete inhibition of mitochondrial swelling in both types of mitochondria (Figs. 1a, 2a) and full protection of the $\Delta\Psi$ drop (Figs. 1b, 2b). As a control, Figures 1 and 2 also show the inhibitory effect of cyclosporin A (CsA), the typical inhibitor of MPT. The incomplete inhibition by CsA observed in RBM, as also reported elsewhere [23, 24], is most likely due to a reduced sensitivity of cyclophilin in brain towards CsA [25]. The inhibitory effect by PF9601N on MPT is dose dependent, with an IC_{50} value of 8.8 μM in RLM (95% confidence interval range 6.1–10.5 μM) (Fig. 1a, inset) and 26 μM in RBM (95% confidence interval range 19.9–28 μM) (Fig. 2a, inset). Addition of PF9601N after 6 min of incubation, that is, after the triggering of swelling, also results in inhibition of the phenomenon (Figs. 1a, 2a), demonstrating that the effect of the drug is not attributable to inhibition of both Ca^{2+} and P_i transport. For comparison, the effects of the propargylamines and desmethyldeprenyl (the main metabolite of deprenyl), at the same concentration of 50 μM, on the swelling of both RLM and RBM are also shown in Figs. 1a and 2a, respectively. Deprenyl does not provide any protection, whereas in both

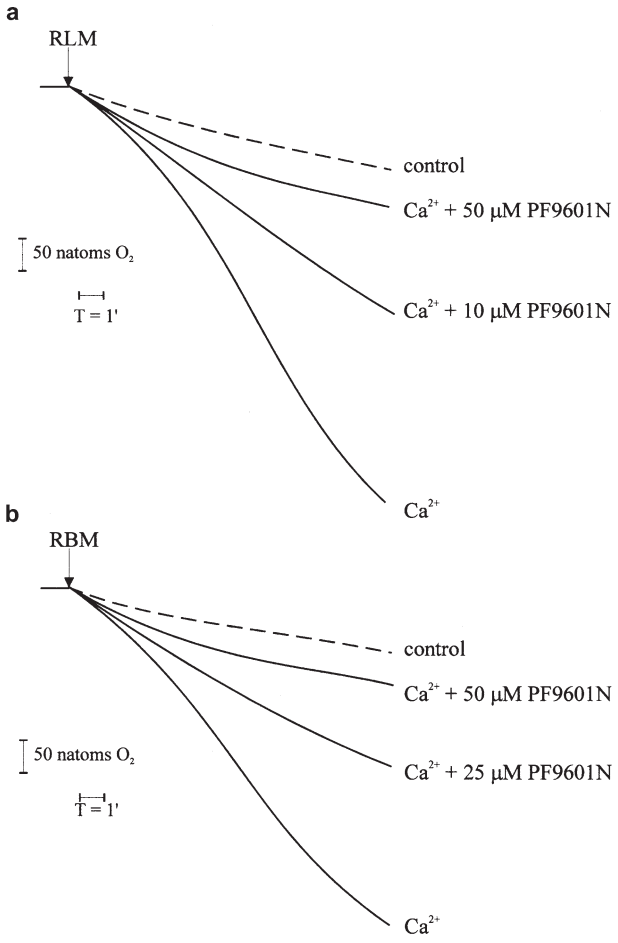


Figure 3. Inhibitory effect of PF9601N on enhanced oxygen uptake induced by Ca^{2+} in RLM (*a*) and RBM (*b*). RLM and RBM were incubated in standard medium. Where indicated 50 μM Ca^{2+} was present. PF9601N was present at the indicated concentrations. Assay was performed four times, with comparable results.

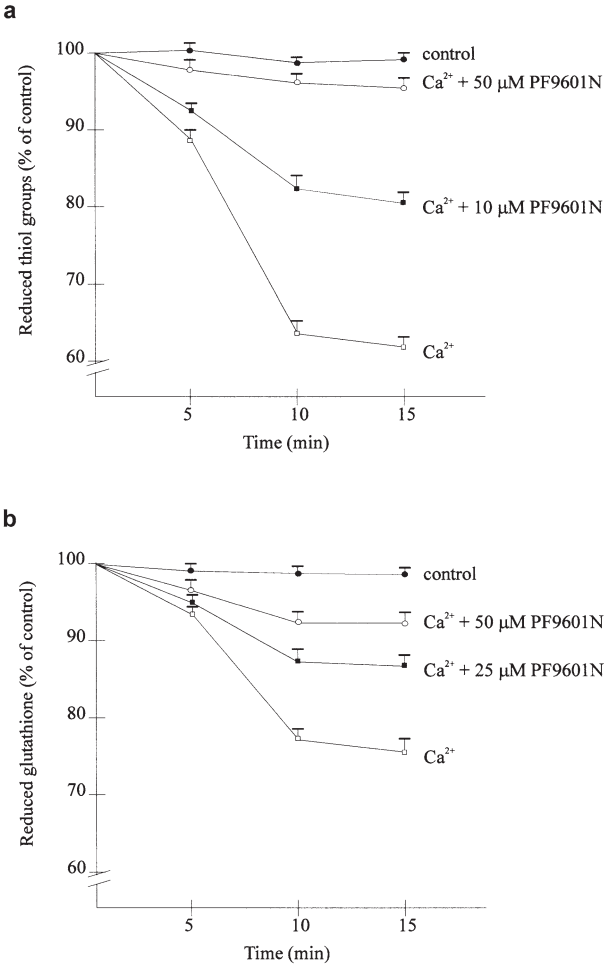


Figure 4. PF9601N prevents oxidation of thiol groups (*a*) and glutathione (*b*) induced by Ca^{2+} in RLM. RLM were incubated in standard medium, as described in Fig. 3. PF9601N was present at the indicated concentrations. Values are means ± SD of seven different experiments. The contents of reduced thiol groups and reduced glutathione were 72.1 ± 0.72 and 2.73 ± 0.02 nmol/mg protein, respectively.

RLM and RBM desmethyldeprenyl exhibits strong inhibition, although it is not complete when compared with PF9601N. For a degree of inhibition similar to that observed with PF9601N, concentrations of 700 μM deprenyl or 70 μM desmethyldeprenyl, must be used (Figs. 1a and 2a). At 50 μM, PF9601N also completely prevents the stimulation of oxygen uptake (Fig. 3a, 3b) and maintains the levels of reduced thiol groups and reduced glutathione at 95% and 92%, respectively, in RLM (Fig. 4a, 4b) and 96% and 98% in RBM (Fig. 5a, 5b). Indeed, the drug almost completely prevents pyridine nucleotide oxidation (Fig. 6a, 6b). If PF9601N is present at concentrations very close to the IC_{50} values (10 μM in liver and 25 μM in brain), it exhibits an appreciable inhibition of $\Delta\Psi$ collapse (Figs. 1b, 2b) and of the stimulation of O_2 uptake and oxidation of thiols, glutathione and pyridine nucleotides (Figs. 3–6); according to what is observed in mitochondrial swelling (Figs. 1a and 2a: insets). The opening of the transition pore is also closely related to the release of some mitochondrial protein such as cyt c [10], due to outer membrane rupture as result of mitochondrial swelling. The Western blots of Fig. 7 show that Ca^{2+} plus P_i induces the loss of a considerable amount of cyt c from RLM (Fig. 7a, upper gel) and RBM (Fig. 7b, upper gel), measured after 15 min of incubation. The same panels also show that, in the presence of 50 μM PF9601N, cyt c is almost completely retained by RLM and RBM, whereas at 10 μM in liver and 25 μM in brain, cyt c is only partially retained. In the absence of Ca^{2+}, no effect by PF9601N is observable. The lower gels in both Fig. 7a and b show the time-dependent release of cyt c from RLM and RBM, respectively, induced by Ca^{2+} plus P_i. It should be pointed out that the drug alone (in the absence of Ca^{2+}) does not have any effect on inner membrane integrity (see Figs. 1, 2). Experiments performed in an isotonic KCl medium gave similar results (data not reported).

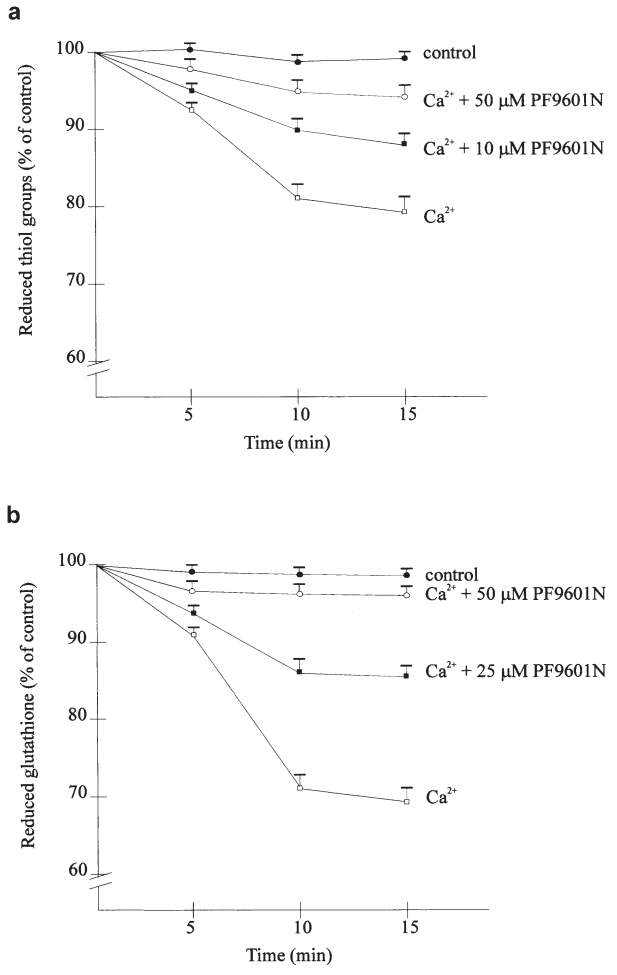


Figure 5. PF9601N prevents oxidation of thiol groups (*a*) and glutathione (*b*) induced by Ca^{2+} in RBM. Incubation conditions as in Fig. 3.Values are means ± SD of five different experiments. The content of reduced thiol groups and reduced glutathione were 38.4 ± 0.41 and 5.25 ± 0.05 nmol/mg protein, respectively.

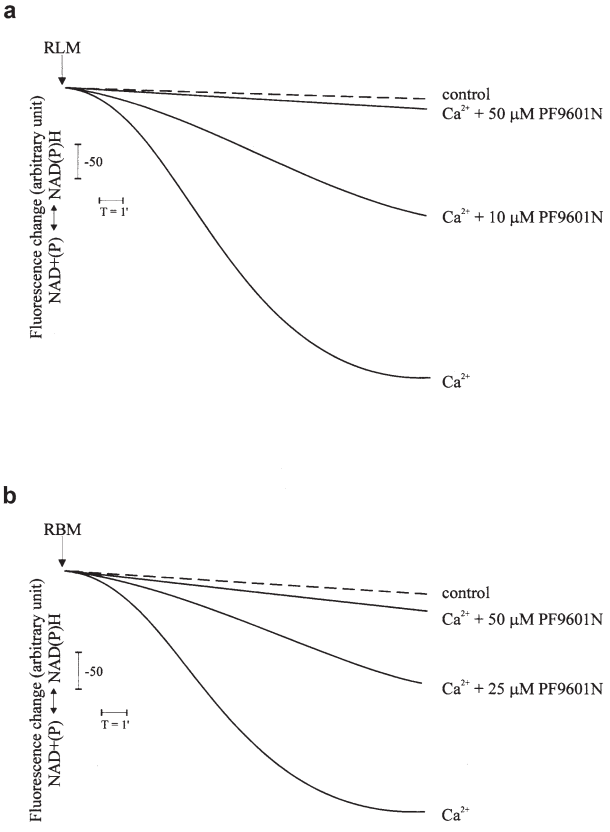


Figure 6. PF9601N prevents oxidation of pyridine nucleotides in RLM (*a*) and in RBM (*b*). Incubation conditions as in Fig. 3. The curve are representative of four similar experiments.

Discussion

The above experimental data clearly demonstrate that the indolakylamine PF9601N is able to prevent induction of MPT in RLM and RBM subjected to the action of supraphysiological concentrations of Ca^{2+} in the presence of P_i, in that, at 50 µM concentration, the drug can block mitochondrial swelling (Figs. 1a, 2a), $\Delta\Psi$ drop (Figs. 1b, 2b) and also cation efflux (results not reported). It also prevents the increase in oxygen uptake (Fig. 3a, 3b) and the oxidation of glutathione, thiol groups and pyridine nucleotides in RLM (Figs. 4a and b, 6a) and RBM (Figs. 5a, b; 6b). This behavior on the part of PF9601N is typical of an inhibitor of pore opening. It should be noted that the concentration used, 50 µM, is very close to those used to protect PC12 cultured cells (50–100 µM) [5] and human neuroblastoma SHSY5Y cells (10 µM) [6] against the oxidative stress induced by dopamine. Indeed, the neuroprotective effect by PF9601N, injected in rat at 60–120 µmol/kg body weight, on the neurodegeneration of nigral neurons induced by oxidative stress has also been observed [3, 4].

The MPT induced by Ca^{2+} and P_i may be explained by considering the sequential model proposed by Davidson and Halestrap [26], in which transition pore opening is the result of conformational changes taking place on adenine nucleotide translocase (AdNT). This model predicts that both P_i and pyrophosphate (probably produced by a proton-translocating pyrophosphatase [27] activated by Ca^{2+} [24]) replace ADP and ATP, respectively, also bound to AdNT. This action transforms AdNT into a channel, allowing electrogenic uptake of K^+. The binding of Ca^{2+} to specific inner sites of AdNT then transforms the K^+ channel into a unselective transition pore sensitive to CsA [28]. However, as reported by others, the opening of the pore also requires the oxidation of critical thiol groups located on AdNT [29] and of glutathione and pyridine nucleotides [30]. The increase in oxygen uptake (Fig. 3) and the observed oxidation of these compounds (Figs. 4–6) are in agreement with the above statement. Also, although if the onset of MPT is more extensive and rapid in liver than in brain, PF9601N is able to completely prevent the induction of the phenomenon in both types of mitochondria. However, the IC_{50} is notably higher in

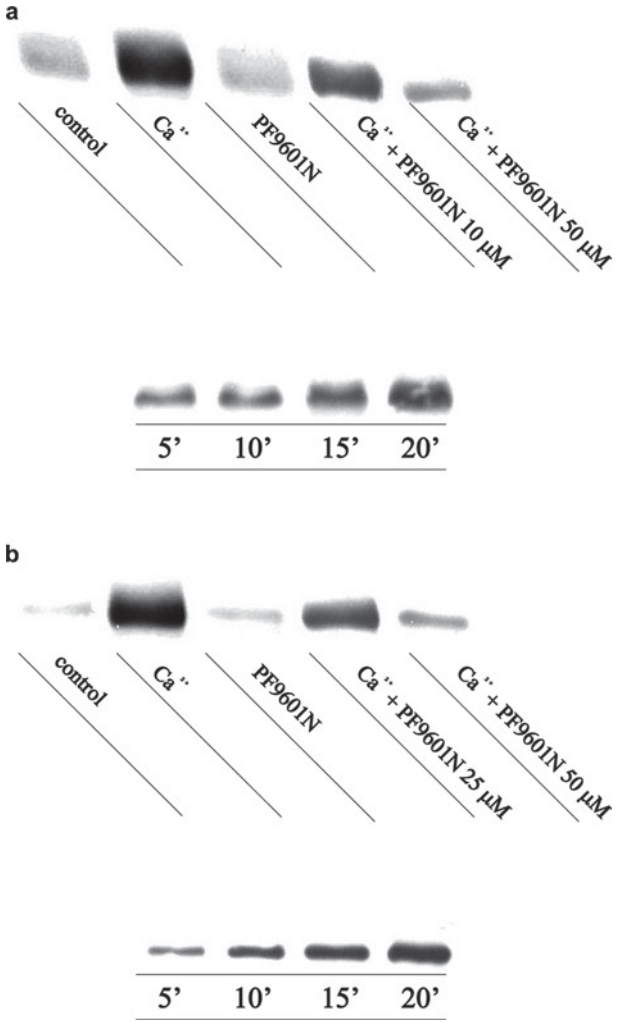


Figure 7. Release of cyt c induced by Ca^{2+} and prevention by PF9601N in RLM (*a*) and RBM (*b*). Results of Western blotting of supernatant fractions shown. RLM were incubated in standard conditions indicated in Materials and methods. Where indicated 50 μM Ca^{2+} was present. PF9601N was present at the indicated concentrations. Upper gels in both panels: determinations after 15 min of incubation. Lower gels in both panels: time-dependent release of cyt c. Three other experiments with both RLM and RBM gave almost identical results.

brain, suggesting that this pathway does not allow the drug to reach the site of its action easily. A different pathway for the drug is supported by the observation that, when it is added after induction of swelling, the potency and rate of action are the same in the two mitochondrial preparations (Figs. 1a, 2a). In this case, pore opening allows the drug to reach its site of action more easily, rendering its effects on both types of mitochondria almost identical. Concentrations very close to the IC_{50} values (10 μM in liver and 25 μM in brain) exhibit expected inhibition of $\Delta\Psi$ collapse (Figs. 1b, 2b), O_2 uptake stimulation (Fig. 3), and oxidation thiols, glutathione, and pyridine nucleotides oxidation (Figs. 4–6), by approximately 50%. This means that the target of PF9601N is closely related to these alterations. As regard the mechanisms by which Ca^{2+} induces the generation of the ROS responsible for this oxidative stress, it has been proposed that, by interacting with the membrane cardiolipins, the cation induces a change in the motility of the ubiquinone pool present in the bulk hydrophobic phase of the membrane. This affects electron flux in the respiratory chain, favoring accumulation of semiquinone radicals and the subsequent generation of hydrogen peroxide and hydroxyl radicals [31].

As previously observed also for other compounds containing amino groups (*e.g.* monoamines, propargylamines, polyamines, carnitine, tetracaine) [9], PF9601N is protonated at physiological pH and can act as a monovalent cation able to inhibit MPT. A previous explanation for this general effect exhibited by cationic organic compounds took into account the fact that these molecules are inhibitors of K^+ channels and that MPT exhibits the archetypal properties of these channels [26]. The interaction of PF9601N with critical amino acid residues, present in the structures forming the pore, may be responsible for the observed inhibition. This hypothesis is strongly supported by the observation that the drug is able to block the phenomenon after pore opening (Figs. 1a, 2a).

One mechanism that may explain the protective effect of PF9601N concerns its chemical structure (see scheme 1). PF9601N is a tryptamine derivative, having the same acetylene group as L-deprenyl, but with a secondary amino group instead of a tertiary one. The observation that desmethyldeprenyl, which also possesses a secondary protonated amino group, is much more efficient than deprenyl (Figs. 1a, 2a) reveals the importance of this amino group in protection against MPT. This higher effectiveness is due to the different basic properties and consequent degrees of protonation of amino groups, secondary > tertiary, which allows desmethyldeprenyl to establish stronger interactions with pore-forming structures than deprenyl. The presence of a methyl, very close to the protonated nitrogen of desmethyldeprenyl (and also deprenyl) is most probably responsible for its lower efficacy with respect to PF9601N (Figs. 1a, 2a).

However, it has also recently been reported that indolalkylamine derivatives act as antioxidant agents [6], *i.e.* that the targets of PF9601N are ROS produced by mitochondria. This property must also be considered in the observed protective effect on MPT induction. In addition, the preventive effect of PF9601N on the oxidative stress induced by Ca^{2+} and P_i is not due to inhibition of the drug on MAO B activity, as this enzyme is not involved in the process of pore opening. This observation demonstrates the more generalized protective effect of PF9601N involving the inhibition of MPT. As oxidative stress is responsible for the damaging effects in mitochondria and cells, and in *in vivo* conditions, and considering that ROS are mainly produced in mitochondria [7], the effects observed *in vitro*, reported here, may be correlated with

those observed in whole cells [5, 6] and in *in vivo* [3, 4]. This hypothesis is strongly supported by the observation that the concentrations of PF9601N able to protect mitochondria, cultured cells and animals are very close to one another (see above). Inhibition of cyt c release induced by Ca^{2+} plus P_i (Fig. 7) suggests the potential anti-apoptotic action of PF9601N.

The IC_{50} values for the inhibition of MPT by PF9601N are 8.8 μM in RLM (Fig. 1a, inset) and 26 μM in RBM (Fig. 2a, inset). It should be pointed out that the concentration of PF9601N able to induce almost complete inhibition of mitochondrial swelling is more than one order of magnitude lower than that of the propargylamine L-deprenyl, another well-known MAO B inhibitor (Figs. 1a, 2a), which has similar effects on RLM [32]. This property, when considered together with the lack of the amphetamine moiety in the molecule, makes PF9601N a more efficient compound than L-deprenyl in protecting bioenergetic functions, not only of isolated RLM or RBM but also, most probably, of organelles *in situ*. This is because generation of the metabolites of L-deprenyl, *i.e.* amphetamine and methamphetamine, which may have adverse and severe side effects, is avoided. It should be emphasized that PF9601N is also more effective than several other monoamines in protecting mitochondria against MPT. For example, tyramine and benzylamine act at a concentration of 1 mM [17, 33]. Other propargylamines, pargyline and clorgyline, have a partial effect at 500 and 50 μM, respectively [17]. Carnitine acts partially at 750 μM [34]. However, several diamines such as putrescine, cadaverine [35] and tetracaine [36] are less effective than PF9601N. Taking into account that PF9601N has similar effects also on heart and kidney mitochondria (data not reported), the above results do not show any particular tissue specificity on the part of PF9601N. In conclusion, this study reveals the possibly protective role of PF9601N against diseases resulting from the toxicity induced by oxidative stress in mitochondria.

Preliminary results obtained in our laboratory, showing that PF9601N prevents apoptosis in neuroblastoma cells and hepatocyte cultures, allow us to make some hypotheses. It should be noted that, in Parkinson's disease, mitochondrial dysfunction as a consequence of oxidative stress and opening of the mitochondrial pore induces neuronal death by an apoptotic process [37]. Furthermore, with the opening of the transition pore, liver mitochondria undergo significant *in vivo* alterations in morphology, *i.e.* from spherical organelles to tubovescicular structures [38]. This leads to the pleomorphic mitochondria typical of liver diseases and cirrhosis [39]. Inhibition of mitochondrial pore opening by PF9601N confirms that this new molecule is a promising drug for use in treating neurodegenerative and liver diseases.

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