Mitochondria as a Target for Neurotoxins and Neuroprotective Agents

SERGEY O. BACHURIN,[a] ELENA P. SHEVTSOVA,[a] ELENA G. KIREEVA,[a] GREGORY F. OXENKRUG,[b,c] AND SERGEY O. SABLIN[d]

[a]*Institute of Physiologically Active Compounds, Chernogolovka, Russia*

[b]*Department of Psychiatry and Pineal Research Laboratory, St. Elizabeth's Medical Center, Boston, Massachusetts, USA*

[c]*Department of Veterans Affairs Medical Center, Providence, Rhode Island, USA*

[d]*Selena Pharmaceuticals, Inc., San Francisco, California, USA*

ABSTRACT: Mitochondrial permeability transition pores represent a multiprotein complex that includes components of both inner and outer membrane. The pores regulate transport of ions and peptides in and out of mitochondria, and their regulation is associated with a general mechanism for maintaining Ca^{2+} homeostasis in the cell and apoptosis. Various pathologic factors may induce a pathologic activation of the permeability transition and an irreversible opening of mitochondria pores. This event is a major step in the development of neurotoxicity and neurodegeneration. This paper explores the effect of MPP^+ and β-amyloid fragment 25–35, neurotoxins that are known to generate Parkinson's-like syndrome and Alzheimer's disease, on the regulation of the mitochondrial pores. Both neurotoxins induce opening of mitochondrial pores, which is prevented by cyclosporin A, a specific inhibitor of the permeability transition. The effect of MPP^+ and β-amyloid may be also prevented by an endogenous precursor of melatonin, *N*-acetylserotonin, by an anti-Alzheimer's medication tacrine, and by dimebon, which is in development as an agent for the therapy of Alzheimer's disease and other types of dementia. The paper illustrates that the effect on mitochondrial pores is an important aspect of the mechanism of neurotoxicity. Substances that may prevent opening of mitochondrial pores induced by neurotoxins may preserve the mitochondrial function and, thus, may have potential as neuroprotective agents.

KEYWORDS: mitochondria; permeability transition pores; *N*-acetylserotonin; tacrine; dimebon; neurodegeneration; apoptosis

INTRODUCTION

A general deficit of the biological energy and a malfunction of brain mitochondria is the earliest and the most specific feature of aging and a wide range of neurodegenerative disorders.[1,2] It is also known that many so-called mitochondrial diseases are always accompanied with brain malfunctions.[3]

Address for correspondence: Sergey O. Sablin, Ph.D., Selena Pharmaceuticals, Inc., 167 Skyview Way, San Francisco, CA 94131, USA. Voice: 415-990-4761.
sablin@selenapharma.com

Ann. N.Y. Acad. Sci. **993: 334–344 (2003).**

One general characteristic of aging and neurodegeneration is an increase in the number of neural cells showing signs of an apoptotic degeneration. Recently it was found[4] that a key role in apoptotic processes is attributable to so-called mitochondrial permeability transition pores (PTP), which provide transport in and out of mitochondria for both calcium ions and for compounds with molecular weight of up to 1.5 kDa. It was proposed that PTP represent a multiprotein complex, which is located at the contact sites of the mitochondrial membrane and which includes components of both the inner and the outer membrane.[5] An outer membrane fragment of PTP includes the so-called *porin*, a voltage-dependent anion channel, antiapoptotic proteins of the Bcl-2 family, and, probably, a peripheral benzodiazepine receptor. The inner membrane fragment of PTP contains an adenine nucleotide translocator (ANT) and cyclophilin, which may interact with proapoptotic proteins of the Bax family. It is likely that the enzymes creatine kinase and hexokinase also participate in the regulation of PTP.

In the *normal* (low-conductive) state of PTP at physiologic concentration of cytosol calcium ions, opening and closing of PTP is reversible and is regulated by the membrane potential. This function of PTP provides calcium homeostasis in the cell. The influx of calcium ions into mitochondria is accompanied by the simultaneous efflux of protons, resulting in an increase of pH in the mitochondrial matrix, followed by opening of PTP. This ion flux also results in a collapse of both proton gradient and a membrane potential, a collapse of Ca^{2+} reuptake, and acidification of the mitochondrial matrix. The latter event induces closing of PTP. Functioning of the mitochondrial respiratory chain restores the proton gradient that allows calcium ions to reenter mitochondria, and so forth. Thus, under *normal* physiologic conditions, mitochondria serve as a buffer to compensate for the *excessive* calcium. In pathology, an excessive Ca^{2+} influx into the cell (e.g., via the NMDA receptor system) leads to the accumulation of calcium ions inside mitochondria and triggers a massive generation of ROS, which induces degradation of Ca^{2+}-dependent ATPase. This process, in turn, decreases the ability of the cell membrane to eliminate excessive calcium from the cell and amplifies an additional increase in intramitochondrial calcium concentration. A critical overload of mitochondria with Ca^{2+} induces depolarization of the mitochondrial membrane and an irreversible opening of PTP. These changes are accompanied by the exit from mitochondria of calcium ions and macromolecular compounds, such as caspases activators, cytochrome c, and Apaf-1 factors. Together, these reactions are thought to predetermine cell death.

Inhibition of the mitochondrial Ca^{2+} uptake and/or blocking of PTP may protect cells against the development of apoptosis (and necroses) in the presence of pathologic factors, such as excitotoxins and oxidants.[6] The Ca^{2+}-dependent activation of PTP in brain mitochondria was shown to be enhanced with age,[7] and may play an important role in the development of age-related neurodegenerative disorders.

In this paper we report on a study of the effect of neurotoxins MPP^+ and β-amyloid fragment 25–35 (both of which are used to model Alzheimer's disease and Parkinson disease) on the PTP state. We also investigated how the anti-Alzheimer's agents Tacrine and Dimebon, and the endogenous antioxidant and neuroprotector, *N*-acetylserotonin (NAS), influence regulation of the PTP function.

MATERIALS AND METHODS

The β-amyloid fragment (25–35) was purchased from Bachem; Dimebon was provided by NPO Organika (Novokuznetsk, Russia). Tacrine and all other reagents were purchased from Sigma.

Liver mitochondria were isolated and purified from liver of Wistar rats using a standard procedure[8] in a 5 mM HEPES buffer, pH 7.4, containing 210 mM mannitol, 70 mM sucrose, and 1 mM EDTA (buffer A). Because mannitol binds hydroxyl ions, it was omitted in the assay buffer (buffer B), when mitochondria swelling and the intensity of lipid peroxidation were monitored simultaneously. The final centrifugation and resuspension of mitochondria were performed in buffer A or buffer B in which EDTA was omitted (A-EDTA and B-EDTA, respectively). Brain mitochondria were isolated and purified in Percoll gradient according to the method of Sims *et al.*[9] in 10 mM HEPES buffer, pH 7.4, containing 0.3 M glucose and 1 mM EDTA at 4°C.

Opening of PTP was indicated by mitochondria swelling, which resulted in a decrease in optical density of the mitochondria suspension. The absorbance was monitored using Beckman DU 640 spectrophotometer at 540 nm and constant temperature 25°C in thermostated cuvette equipped with a continuous stirring device. The reaction mixture contained buffers A-EDTA or B-EDTA, 0.8 μM rotenone, 5 mM succinate, and mitochondria suspension diluted to give a 1 mg/mL protein in the cuvette. The reaction was initiated by adding an agent that triggered PTP opening. The rate of mitochondria swelling was calculated as a maximum gradient of change in the absorbance at 540 nm, and expressed in *d*A540/min/mg of protein.

Lipid peroxidation in mitochondrial membranes was followed by an accumulation of substances that reacted with thiobarbituric acid, monitored spectrophotometrically according to a procedure described by Erdahl and coauthors.[10]

All experiments were repeated with at least three different mitochondria preparations. The figures illustrate results from selected experiments.

RESULTS AND DISCUSSION

Effect of Neurotoxins MPP and β-Amyloid Fragment (25–35) on the Opening of Mitochondrial Pores

Mitochondrial response to the presence of any agent that causes pore opening is characterized by two parameters, the lag period (interval between the moment when the triggering agent is introduced and the time when pores begin to open) and the maximum slope (which reflects the rate of PTP opening). Opening of mitochondrial pores results in the massive entry of sucrose and other substances into the mitochondrial matrix. This causes swelling of mitochondria and, as a result, a decrease in absorbance of the mitochondrial suspension.

In the presence of calcium ions or phosphate ions in concentrations that do not induce the PTP opening, a neurotoxin MPP^+ induced a decrease in the absorbance of the suspension of isolated mitochondria (see FIGURE 1). Introduction of a specific PTP inhibitor, cyclosporin A, into the incubation media of the 2 μM solution prevented the mitochondria swelling caused by MPP^+. Although the response of the mitochondrial pores to the introduction of MPP^+ was concentration dependent, this

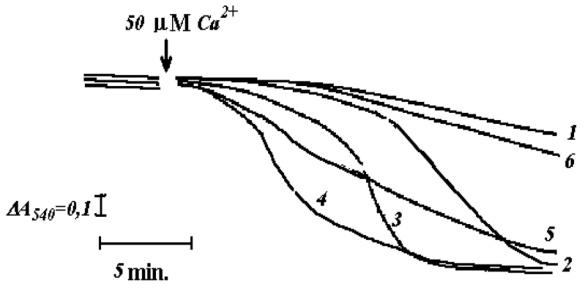

FIGURE 1. Effect of MPP^+ on Ca^{2+}-induced PTP opening. Mitochondria were preincubated for five minutes in buffer A containing glutamate, malate, and 0.2 mM potassium phosphate in the presence of various concentrations of MPP^+: (1) control; (2) 0.075 mM MPP^+; (3) 0.15 mM MPP^+; (4) 0.3 mM MPP^+; (5) 0.6 mM MPP^+; and (6) 0.6 mM MPP^+ and 2 μM cyclosporin A.

dependence is rather complex and, at present, is difficult to interpret. The effect of MPP^+ in concentrations of up to 500 μM is characterized by a progressive increase in the rate of PTP opening and a reduction in the lag period. No effect of MPP^+ on the intensification of lipid peroxidation was found at these concentrations, a result that is in agreement with other observations.[11] Additional increase in the MPP^+ concentration resulted in a failure of a general mitochondrial response, which was expressed as an additional reduction in the lag period, but in a decrease in the maximum slope. It also resulted in an intensification of lipid peroxidation. Our observations agree well with the report by Cassarino *et al.,*[12] who used brain and liver mitochondria in their experiments.

The β-amyloid fragment 25–35 also exerted a pronounced effect on the rat liver mitochondria. It remarkably induced the mitochondria response (see FIGURE 2),

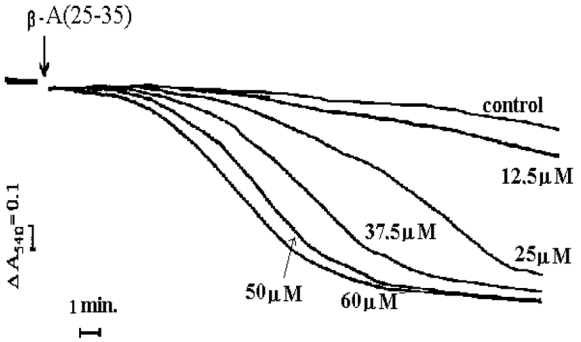

FIGURE 2. The β-amyloid fragment 25–35 induces opening of mitochondria pores even when no calcium ions or phosphate ions are added to the reaction mixture. Mitochondria were added to the incubation buffer containing succinate and rotenone at 25°C. The reaction was initiated by adding varied concentrations of β-amyloid as indicated in the figure.

which was enhanced by calcium or phosphate ions. The induction was dose dependent and was inhibited by cyclosporin A. A similar effect of β-amyloid (25–35) on PTP was observed in experiments with brain mitochondria. It should be noted, however, that brain mitochondria were somewhat less susceptible to β-amyloid, as well as to other inducers of PTP, a result that is consistent with observations by others.[13] This fact may be associated with either a higher heterogeneity of brain mitochondria, or a heterogeneity of the starting material, or a potential presence of synaptosomes in the preparation of brain mitochondria.

Since induction of PTP opening by MPP^+ and by bA(25–35) was dose dependent and was prevented by cyclosporin A, it is likely that both neurotoxins have a specific effect of the regulation of PTP. This effect on PTP may be one of the earliest toxic effects of the β-amyloid peptide in the pathogenesis of Alzheimer's disease. The fact, that toxic forms of the amyloid peptide may be generated inside the neuron, also suggests that mitochondria may be one of the primary targets of the cytotoxic activity of β-amyloid.

Amyloid fragment bA(25–35) significantly induced lipid peroxidation in mitochondria. However, although specific PTP inhibitor cyclosporin A almost completely inhibited mitochondrial swelling caused by β-amyloid (25–35), its effect on the formation of substances that react with thiobarbituric acid was insignificant (data not shown). It is known that antioxidants suppress oxidative stress and lipid peroxidation in the cell, but do not have an effect on cell death.[14,15] Apparently, the induction of opening of mitochondrial pores by β-amyloid is only partially associated with its ability to cause lipid peroxidation. Therefore, agents that have antioxidant properties and that also prevent the induction of PTP opening may reduce the toxic effect of β-amyloid and other types of neurotoxins on mitochondria. These agents may be of an interest as a new class of neuroprotectors. To explore this concept, we studied the effect of several compounds, *N*-acetylserotonin, tacrine, and dimebon, on PTP function. These compounds are either on the market or are in development as agents for the therapy of Alzheimer's disease that have a potential neuroprotective effect.

Effect of N-*Acetylserotonin on the Function of PTP*

Recently it was demonstrated that *N*-acetylserotonin (NAS), a metabolic precursor of melatonin, displayed more pronounced antioxidative properties than melatonin itself.[16] In addition, NAS showed an anti-aging effect[17] and a neuroprotective effect.[18]

FIGURE 3 illustrates that NAS suppressed the formation of products of lipid peroxidation caused by either butylhydroperoxide (FIG. 3A) or by iron ions (FIG. 3B) in isolated rat liver mitochondria. A dose-dependent inhibition of lipid peroxidation observed in our experiments shows good correlation with results from other laboratories. Karbownik and coauthors[19] studied the effect of NAS on lipid peroxidation in microsomal liver membranes and testis membranes, where lipid peroxidation was generated by $FeCl_2$, ADP, or NADPH. In the same concentration range, NAS has a stabilizing effect on membranes. It does not alter the membrane state in the absence of the oxidative stress, but it prevents the membrane fluidity induced by the oxidative stress.[20]

The effect of NAS on PTP is rather complex. In pharmacologic concentrations, at which NAS inhibits lipid peroxidation, NAS demonstrates only marginal protective effect on the opening of PTP generated by various inductors. At NAS concentrations between 1 μM and 100 μM, the inhibition of PTP is unstable and insignificant (not

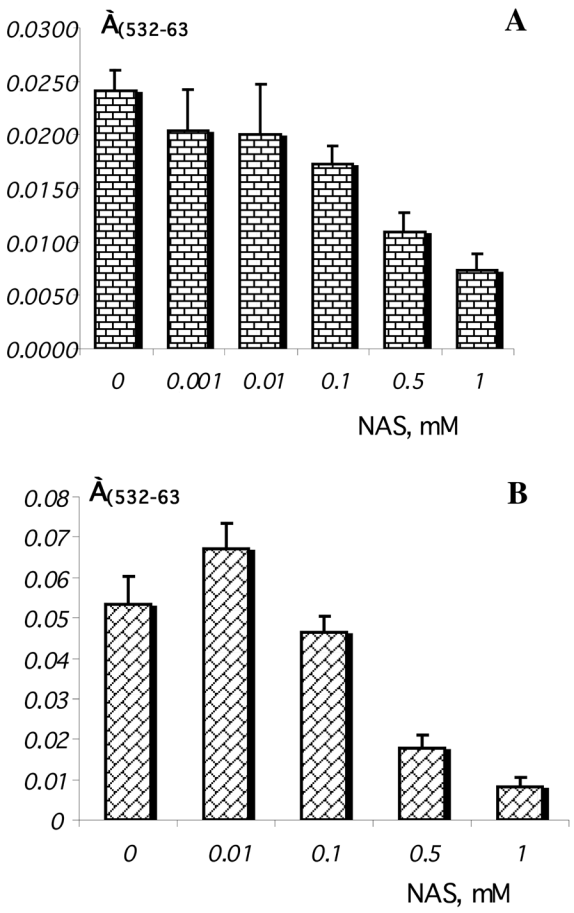

FIGURE 3. The melatonin precursor, *N*-acetylserotonin, suppresses lipid peroxidation stimulated either by **(A)** 0.2 mM *tert*-butylhydroxyperoxide and 0.05 mM calcium ions or by **(B)** 0.0375 mM $FeSO_4$ and 0.05 mM calcium ions. Rat liver mitochondria were added in buffer B to give concentration of 1 mg of protein/mL, and were incubated for 30 min in the presence of various concentrations of NAS and either *tert*-butylhydroxyperoxide or iron ions. The formation of substances that reacted with thiobarbiturate was measured as described in METHODS.

more than 20%). Moreover, at concentrations in excess of 100 µM in the presence of mannitol, NAS caused a decrease in the absorbance of the mitochondria suspension that was independent of the presence of cyclosporin A. The mechanism of this phenomenon is not clear. Apparently, it is not associated with the opening of PTP because it is independent of the presence of the specific inhibitor cyclosporin A.

Physiologic concentrations of melatonin and its precursors and its metabolites do not exceed nanomolar range.[21] In a very narrow concentration range, between 5 and

50 nM, NAS suppressed opening of PTP induced by calcium (100 μM) and by phosphate (0.5 mM). The concentration range, where NAS was active toward PTP, was not consistent for different mitochondria preparations and, occasionally, could not be detected at all. However, the inhibition of PTP opening by NAS, which was induced by MPP^+, was observed in the same nanomolar concentration range (see FIGURE 4) and was consistent for all the mitochondria preparations.

Melatonin protects against the toxicity induced by MPP^+.[22] It is believed that the effect of melatonin is associated with its ability to prevent inhibition by MPP^+ of the Complex I of the mitochondrial respiration chain, rather than with its antioxidant properties. The same is also likely to be true for NAS. Thus, inhibition of PTP opening may be one of the physiologic mechanisms of the neuroprotective effect of NAS, and, possibly, of melatonin.

Effect of Tacrine on the Function of PTP

Tacrine (Cognex, 9-amino-1,2,3,4-tetrahydroacridine) is a non-specific cholinesterase inhibitor, approved in the United States for the therapy of Alzheimer's disease. Tacrine increases the efficiency of cholinergic neurotransmission and has a number of other properties that contribute to its therapeutic effect. It was previously demonstrated that Tacrine protected cultured neural cells against β-amyloid toxicity,[23] which was not probably associated with its anti-oxidative properties.[24]

It is possible that the cytoprotective effect of Tacrine is associated with its effect on the opening of PTP. In our experiments, Tacrine inhibited Ca^{2+}-induced mitochondria swelling at concentrations between 0.5 mM and 1.2 mM. This concentration

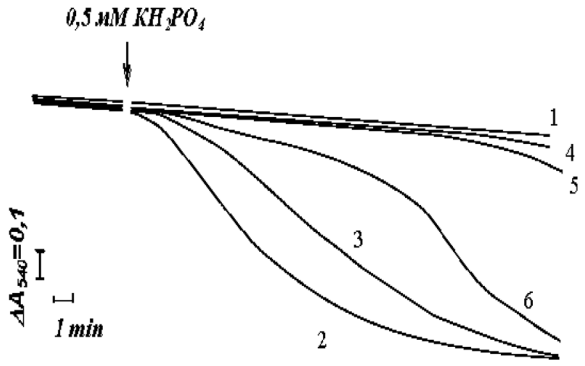

FIGURE 4. The effect of various concentrations of *N*-acetylserotonin on the opening of mitochondrial pores induced by neurotoxin MPP^+. Mitochondria were preincubated for five minutes in buffer A containing glutamate and malate. Then, a solution containing Ca^{2+} was added to give a concentration in the cuvette of 0.05 mM. A solution of MPP^+ was added two minutes later to give a neurotoxin concentration of 0.4 mM in the cuvette. The reaction was triggered in two minutes by adding phosphate to give a concentration of 0.5 mM in the cuvette. The traces on the figure correspond to the control reaction (1), when no neurotoxin was present, the control in the presence of neurotoxin MPP^+ (2), and the reaction in the presence of both neurotoxin (0.4 mM) and NAS in concentrations of 5 nM (3), 10 nM (4), 50 nM (5), and 60 nM (6).

range, however, was essentially higher than concentrations required for the cytoprotective effect (1–50 μM). These observations suggest that the effect of Tacrine on the mitochondria pores may contribute to its cytoprotective effect. However, the main reason is probably associated with the anticholinesterase activity of the drug, since an activation of cholinesterases is linked to the induction apoptosis.[25]

Effect of Dimebon on the Function of PTP

Dimebon, 9-[2-(2-methylpyridyl-5)-ethyl]3,6-dimethyl-1,2,3,4-tetrahydro-γ-carboline, stimulated cognitive functions in the experimental model of AD.[26] Dimebon was successfully tested on Alzheimer's patients in clinical trials and was patented as a new agent for the therapy of neurodegenerative disorders.

Dimebon remarkably suppressed opening of PTP induced by phosphate ions, by calcium ions, or by butylhydroxyperoxide (see FIGURE 5). The inductors selected for our experiments generate different intracellular conditions that result in opening of mitochondrial pores. Calcium ion is an absolute prerequisite for PTP opening. It binds to the specific sites of translocase of adenine nucleotides and cyclophiline, thus providing the condition for PTP opening. Phosphate ions induce PTP opening by reducing the ADP concentration in the mitochondria matrix. Butylhydroperoxide

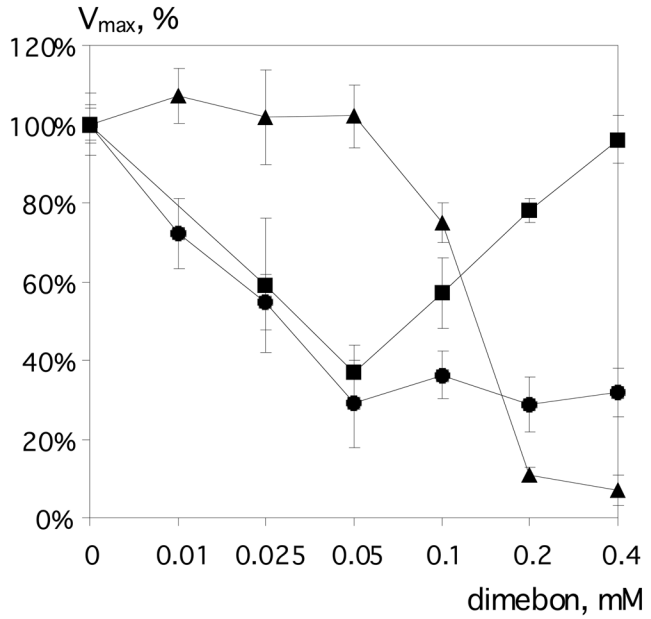

FIGURE 5. The effect of dimebon on the opening of mitochondria pores induced by 0.05 mM Ca^{2+} (●), by 2 mM phosphate ions (▲), or by 0.4 mM *tert*-butylhydroperoxide (■). The ordinate-axis is maximum slope in change of absorbance at 540 nm, which is a function of the rate of mitochondria swelling. Details are described in the text.

is metabolized by the glutathione peroxidase system, which is accompanied by NADPH oxidation. This also results in increasing the probability of PTP opening.

Inhibition of PTP opening by Dimebon dramatically depends on the type of inductor (FIG. 5). Inhibition of the pore opening induced by phosphate ions required higher concentrations of the drug and was complete. The level of inhibition reached its maximum effect at a drug concentration of 50μM when either calcium ions or butylhydroperoxide was used as inductors. Moreover, further increase in the drug concentration resulted in reversing its effect when butylhydroperoxide was used.

Dimebon effectively suppressed lipid peroxidation induced by either butylhydroperoxide or by the β-amyloid fragment 25–35 (data not shown). Suppression of lipid peroxidation and inhibition of PTP opening are not directly related events.[10] Apparently, suppression of lipid peroxidation is not sufficient for the inhibition of PTP opening, since 0.4 mM of dimebon was sufficient to suppress lipid peroxidation, but did not have any effect on PTP opening induced by butylhydroperoxide. In addition, lipid peroxidation does not necessary result in PTP opening, because cyclosporin A completely inhibited pore opening, but only insignificantly suppressed lipid peroxidation in the presence of β-amyloid 25–35.

Thus, dimebon successfully suppressed both lipid peroxidation and PTP opening induced by physiologic inductors. Both of these properties may play a contributing, or even critical, role in the protection of the mitochondrial function and in the overall neuroprotective effect of the drug. The most important observation, however, was the ability of dimebon to prevent PTP opening induced by neurotoxins MPP^+ (see FIGURE 6) and β-amyloid (25–35) (data not shown).

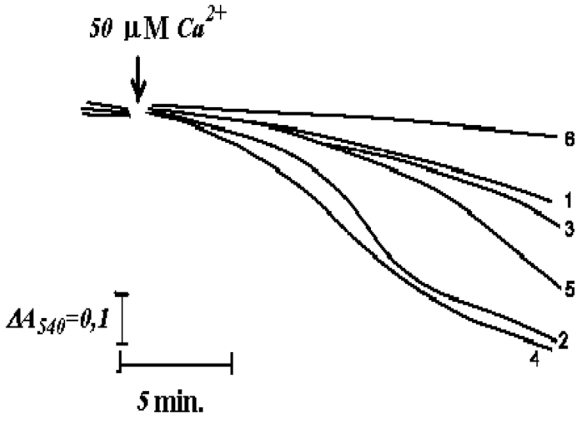

FIGURE 6. Dimebon and cyclosporin A prevent opening of mitochondria pores induced by neurotoxin MPP^+. Mitochondria were preincubated for five minutes in buffer A containing glutamate, malate, and 0.2 mM of phosphate in the presence of various concentrations of MPP^+ and in the presence or in the absence of cyclosporin A or dimebon. The opening of pores was triggered by adding to the reaction mixture a solution containing calcium ions, as indicated in the figure. The traces correspond to the control reaction, when only calcium and phosphate were present (1), and the reaction in the presence of (2) 0.1 mM MPP^+; (3) 0.1 mM MPP^+ and 0.2 mM dimebon; (4) 0.5 mM MPP^+; (5) 0.5 mM MPP^+ and 0.2 mM dimebon; and (6) 0.5 mM MPP^+ and 2 μM cyclosporin A.

In conclusion, the mitochondrial function associated with the regulation of PTP appears to be an important target for neurotoxins, as well as for the substances that may inhibit the effect of such neurotoxins. Compounds that prevent pathologic PTP opening may preserve the mitochondrial function and, thus, may have potential as neuroprotective agents.

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