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Amyloid β-peptide Promotes Permeability Transition Pore in Brain Mitochondria

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In this work the effect of the neurotoxic amino acid sequence, $A\beta_{25-35}$, on brain mitochondrial permeability transition pore (PTP) was studied. For the purpose, the mitochondrial transmembrane potential ($\Delta\Psi m$), mitochondrial respiration and the calcium fluxes were examined. It was observed that $A\beta_{25-35}$, in the presence of Ca^{2+}, decreased the $\Delta\Psi m$, the capacity of brain mitochondria to accumulate calcium and led to a complete uncoupling of the respiration. However, the reverse sequence of the peptide $A\beta_{25-35}$ ($A\beta_{35-25}$) did not promote the PTP. The alterations promoted by $A\beta_{35-25}$ and/or Ca^{2+} could be reversed when Ca^{2+} was removed by EGTA or when ADP plus oligomycin were present. The pretreatment with CsA or ADP plus oligomycin prevented the $\Delta\Psi m$ drop and preserved the capacity of mitochondria to accumulate Ca^{2+}. These results suggest that $A\beta_{25-35}$ can promote the PTP induced by Ca^{2+}.

KEY WORDS: Amyloid β-peptide; permeability transition pore; brain mitochondria; mitochondrial transmembrane potential; calcium fluxes; neurodegeneration.

ABBREVIATIONS: $A\beta$, amyloid beta peptide; AD, Alzheimer's disease; CsA, cyclosporin A; $\Delta\Psi m$, mitochondrial transmembrane potential; GSH, reduced glutathione; PC12, pheochromocytoma cell line; PTP, permeability transition pore; ROS, reactive oxygen species; TPP^+, tetraphenylphosphonium ion.

INTRODUCTION

Amyloid plaque, a pathological marker of Alzheimer's disease (AD), is composed primarily of amyloid beta peptide ($A\beta$) (Glenner, 1988). $A\beta$ is an amphipathic, 39- to 42-residue peptide, which is derived from the transmembrane amyloid precursor protein by proteolytic cleavage (Selkoe *et al.*, 1995). A smaller, 11-residue fragment of $A\beta$ ($A\beta_{24-35}$) possesses much of the biological activity of the full-length peptide. *In vitro* investigations provided firm pharmacological data on dose- (Pereira *et al.*, 1998; 1999) and conformation-dependent (Pike and Cotman, 1993) neurotoxic potentials of $A\beta$. There are several studies suggesting that oxidative stress and altered energy metabolism are involved in the pathogenesis of AD (Smith *et al.*,

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1991; Behl, 1999). Postmortem studies (Kish *et al.*, 1992) demonstrate a decrease in cytochrome oxidase activity in AD brains. However, the critical step of how Aβ fragments induce cytotoxicity, directly via oxidative mechanisms, is still to be ascertained. According to the model reported by Hensley *et al.* (1994) distortions of the electronic structure of Aβ may result in "radicalization" of the peptide and Aβ itself can act as a free radical. It has been suggested that internalized Aβ, through intimate contacts with intracellular recognition sites, directly damages the membranes of intracellular organelles, such as those involved in the respiratory chain of the mitochondria (Pike and Cotman, 1993). Damage to mitochondrial membranes enhances the production of ROS and compromises neurons by energy depletion. Pereira *et al.* (1999) showed in PC12 cells that, Aβ induces mitochondrial dysfunction and impairment of glycolysis, leading to ATP depletion, by a mechanism involving the generation of ROS.

Mitochondria have a dual importance in the process of oxidative stress. They are probably the most important source of increased free radical production and thus play a crucial role in generating oxidative stress (Boveris *et al.*, 1972; Loschen *et al.*, 1974). On the other hand, mitochondria are one of the possible targets by which oxidative stress exerts its effects on cellular deterioration (Bindoli, 1988), and this is believed to play an important role in neurodegeneration (Beal, 1996). A potentially central factor in cell death in neurodegeneration is the permeability transition pore (PTP) (Kim *et al.*, 1999; Brustovetsky and Dubinsky, 2000). The PTP is a nonselective, high conductance channel that spans the inner and outer mitochondrial membrane (Bernardi *et al.*, 1994; Zoratti and Szabo, 1995). Experimentally, induction of the PTP is characterized by an abrupt swelling and depolarization of membrane potential accompanied by the efflux of mitochondrial calcium, GSH, and NAD(P)H (Bernardi, 1992; Petronilli *et al.*, 1994), all of which are inhibited by the immunosuppressant cyclosporin A (CsA) (Broekemeier *et al.*, 1989).

The aim of the present study was to investigate the effects of $A\beta_{25-35}$ on the PTP of brain mitochondria. Mitochondrial transmembrane potential, mitochondrial respiration and calcium fluxes were the parameters examined.

MATERIALS AND METHODS

Chemicals

$A\beta_{25-35}$ and $A\beta_{35-25}$ were obtained from Bachem AG (Bubendorf, Germany). Protease (Subtilisin, Carlsberg) type VIII was obtained from Sigma. Digitonin was obtained from Calbiochem. All the other chemicals were of the highest grade of purity commercially available.

Isolation of Brain Mitochondria

Brain mitochondria were isolated from male Wistar rats (six weeks) by a method previously described (Rosenthal *et al.*, 1987), with slight modifications. The rats were killed by decapitation, and the brains were rapidly removed and homogenized at 4°C in 10 ml of isolation medium (225 mM mannitol, 75 mM sucrose, 5 mM

Hepes, 1 mM EGTA, 1 mg/ml bovine serum albumin, pH 7.4) containing 5 mg of the bacterial protease type VIII (Subtilsin). Brain homogenates were brought to 30 ml and then centrifuged at 2000*g* for three minutes. The pellet was resuspended in 10 ml of the isolation medium containing 0.02% digitonin (which free mitochondria from the synaptosomal fraction) and centrifuged at 12,000*g* for eight minutes. The mitochondrial pellet was resuspended in 10 ml of medium and recentrifuged at 12,000*g* for ten minutes. Finally, the mitochondrial pellet was resuspended in 300 μl of resuspension medium (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, pH 7.4). Mitochondrial protein was determined by the biuret method calibrated with bovine serum albumin (Gornall *et al.*, 1949).

Membrane Potential (ΔΨm) Measurements

The mitochondrial transmembrane potential was monitored by evaluating transmembrane distribution of TPP^+ (tetraphenylphosphonium) with a TPP^+-selective electrode prepared according to Kamo *et al.* (1979) using a calomel electrode as reference.

Reactions were carried out in a chamber with magnetic stirring in 1 ml of the standard medium supplemented with 3 μM TPP^+. The experiments were started by adding 5 mM succinate to mitochondria in suspension at 0.8 mg protein/ml. After a steady-state distribution of TPP^+ had been reached (ca. two minutes of recording), Ca^{2+} was added and ΔΨm fluctuations recorded. Membrane potential was estimated from the decrease of TPP^+ concentration in the reaction medium as described elsewhere (Moreno and Madeira, 1991). $A\beta_{25-35}$ (50, 100 μM) was incubated five minutes before succinate addition, while 0.85 μM CsA and 2 μg/ml oligomycin plus 1 mM ADP were added for two minutes prior $A\beta_{25-35}$ pre-incubation or mitochondria energization.

Mitochondrial Respiration

Oxygen consumption of isolated mitochondria was monitored polarographically with a Clark oxygen electrode (Estabrook, 1967) connected to a suitable recorder in a 1 ml thermostated water-jacket closed chamber with magnetic stirring. The reactions were carried out at 30°C in 1 ml of the standard medium with 0.8 mg protein. 50 μM $A\beta_{25-35}$ was incubated five minutes before succinate addition. 25 nmol/mg protein FCCP was added one minute after mitochondria energization.

Mitochondrial Calcium Fluxes

Mitochondrial calcium fluxes were measured by monitoring the changes in Ca^{2+} concentration in the reaction medium using a calcium-selective electrode according to previously described procedures (Moreno and Madeira, 1991). The reactions were conducted in an open vessel with magnetic stirring in 1 ml of the reaction medium containing 100 mM sucrose, 100 mM KCl, 2 mM KH_2PO_4, 10 μM EGTA, 5 mM Hepes (pH 7.4), with 2 μM rotenone. Mitochondria (0.8 mg/ml) were energized with

5 mM succinate after one minute of Ca^{2+} addition in the absence and in the presence of $A\beta_{25-35}$ (50, 100 μM) and 0.85 μM CsA or 1 mM ADP plus 2 μg/ml oligomycin.

RESULTS

Effect of $A\beta_{25-35}$ on $\Delta\Psi m$: Induction of PTP

The mitochondrial electric potential ($\Delta\Psi m$) drop is a typical phenomenon that leads to the induction of PTP. In Fig. 1 we can observe the alteration of brain $\Delta\Psi m$ induced by $A\beta_{25-35}$. In control conditions (Fig. 1A), after the energization of mitochondria with succinate, the first and second pulse of 125 nmol Ca^{2+} per mg of protein led to a rapid depolarization followed by repolarization. However, a third pulse of Ca^{2+} led to an irreversible depolarization of mitochondria after a smaller repolarization. The collapse of the $\Delta\Psi m$ is prevented by adding EGTA or oligomycin plus ADP (*data not shown*) which further led to a complete restoration of the

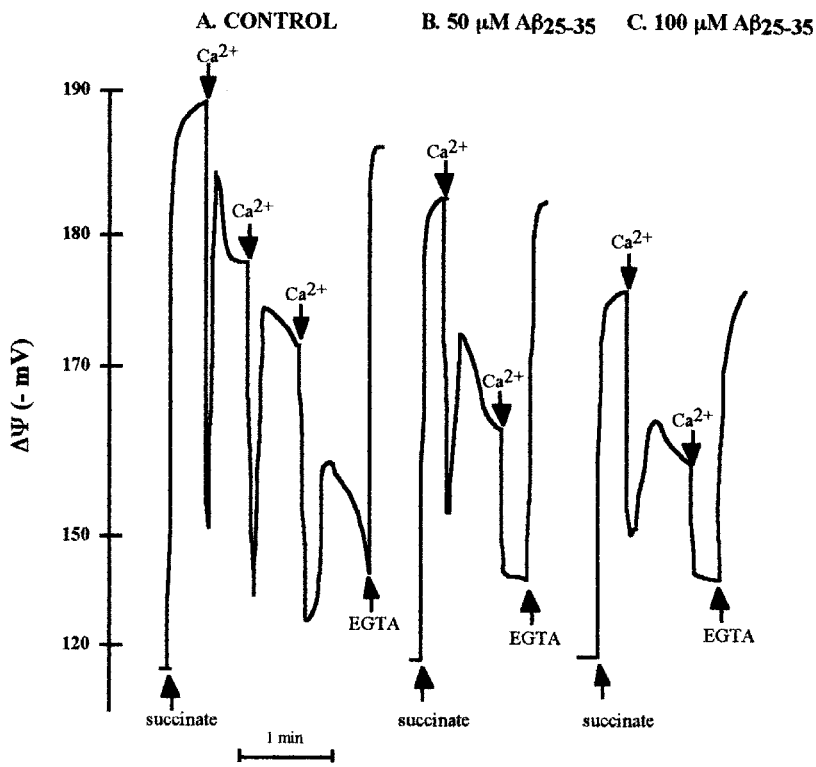

Fig. 1. Effect of $A\beta_{25-35}$ on mitochondrial membrane potential. Freshly isolated brain mitochondria (0.8 mg) in 1 ml of the standard medium supplemented with 3 μM TPP^+ and 2 μM rotenone were energized with 5 mM succinate. A. Ca^{2+} (125 nmol/mg protein) was added one minute after mitochondria energization. B., C. 50 μM $A\beta_{25-35}$ and 100 μM $A\beta_{25-35}$, respectively, were pre-incubated for five minutes at 30°C before mitochondria energization. The traces are typical of four or five experiments.

$\Delta\Psi$m to the state 4 level (e.g., before Ca^{2+} addition). In the presence of Aβ_{25-35} (50 μM and 100 μM) (Fig. 1B, C) a significant decrease of $\Delta\Psi$m, measured after mitochondria energization, occurred. This decrease in $\Delta\Psi$m is more pronounced in mitochondria pre-incubated with 100 μM Aβ_{25-35}. These mitochondria were more susceptible to the amount of Ca^{2+} added; apparently they undergo PTP in the presence of lower Ca^{2+} concentrations (after two Ca^{2+} challenges), when compared to the control mitochondria. The drop in $\Delta\Psi$m and the increased susceptibility to Ca^{2+} were dependent on Aβ_{25-35} concentration.

The reverse sequence of Aβ_{25-35} (Aβ_{35-25}) did not affect the $\Delta\Psi$m as compared with control condition (Fig. 2).

Protection of 1$\Delta\Psi$m Afforded by CsA and Oligomycin Plus ADP: Inhibition of PTP

Pre-incubation (two minutes) of mitochondria with 1 mM ADP plus 2 μg/ml oligomycin afforded complete protection against mitochondrial membrane depolarization, by increasing dramatically the capacity of mitochondria to repolarize the membrane after Ca^{2+} accumulation (Fig. 3).

Additionally, cyclosporin A (CsA), when added two minutes prior to Ca^{2+} or Aβ_{25-35}, afforded a clear protection of mitochondria since it prevents the depolarization induced by of Ca^{2+} when Aβ_{25-35} is present.

Effect of Aβ_{25-35} and FCCP on $\Delta\Psi$m and Respiration

To distinguish between a direct or an indirect action of this peptide on PTP, the effect of 25 nmol/mg protein of FCCP (a mitochondrial respiratory chain uncoupler) (Fig. 4B) was tested. The first pulse of 125 nmol Ca^{2+} per mg of protein led to a rapid depolarization followed by an incomplete repolarization. However, a second pulse of Ca^{2+} led to an irreversible depolarization, a profile similar to that obtained with 50 μM Aβ_{25-35} (Fig. 4A).

Induction of PTP by Aβ_{25-35} is further evidenced by its effect on mitochondrial respiration (Fig. 4). When the mitochondria were pre-incubated for five minutes with 50 μM Aβ_{25-35}, adding 125 μM Ca^{2+} resulted in a complete uncoupling of respiration which was traduced by a continuous stimulation of state 3 respiration. This effect was similar to that exerted by FCCP, in the presence of 125 μM Ca^{2+}.

Effect of Aβ_{25-35} on Calcium Fluxes

Since the mitochondrial collapse of $\Delta\Psi$m, associated to Ca^{2+} overload, is related with PTP opening, experiments were performed to further confirm the Aβ_{25-35} induction of the PTP opening by studying the Ca^{2+} retention by energized mitochondria. Figure 5A shows that isolated brain mitochondria incubated with 50 μM Ca^{2+} (125 nmol/mg protein), in the presence of 2 mM phosphate and energized with succinate, rapidly accumulate Ca^{2+} from the medium and, after a slow release of some of the accumulated Ca^{2+}, they were able to continue to accumulate Ca^{2+}, thus retaining a large portion of the Ca^{2+} present in the medium. In the presence of 100 μM Ca^{2+} mitochondria retained only a small amount of Ca^{2+}. When Ca^{2+} was present in

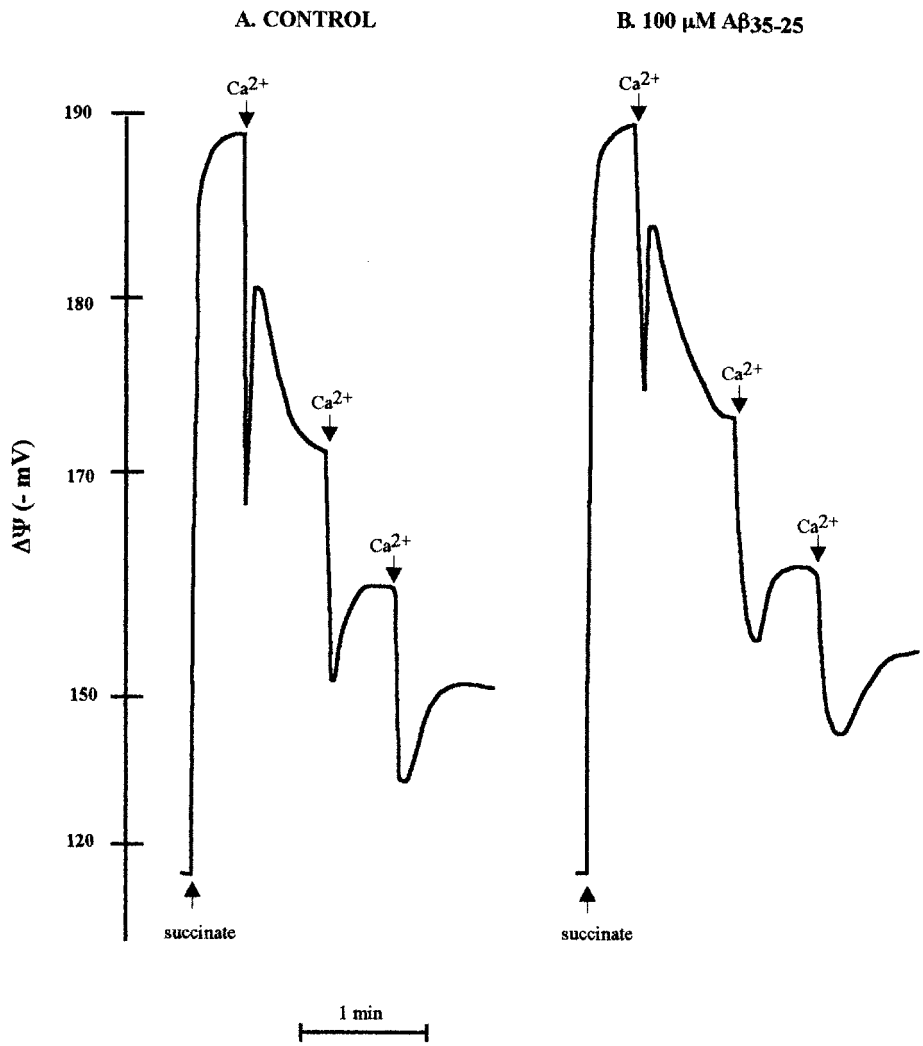

Fig. 2. Effect of $A\beta_{35-25}$ on mitochondrial membrane potential. Freshly isolated brain mitochondria (0.8 mg) in 1 ml of the standard medium supplemented with 3 μM TPP^+ and 2 μM rotenone were energized with 5 mM succinate. A. Ca^{2+} (125 nmol/mg protein) was added one minute after mitochondria energization. B. 100 μM $A\beta_{35-25}$ was pre-incubated for five minutes at 30°C before mitochondria energization. The traces are typical of three experiments.

higher doses (200–300 μM), mitochondria became unable to retain the accumulated Ca^{2+} due to PTP opening. In fact, for 100 μM Ca^{2+}, the addition of CsA or ADP plus oligomycin, restored the ability of mitochondria to store Ca^{2+} by avoiding the PTP opening (Fig. 5A).

The presence of $A\beta_{25-35}$ potentiated the PTP opening (Fig. 5B, C). We also could observe that the effect of $A\beta_{25-35}$ on calcium fluxes was concentration-dependent,

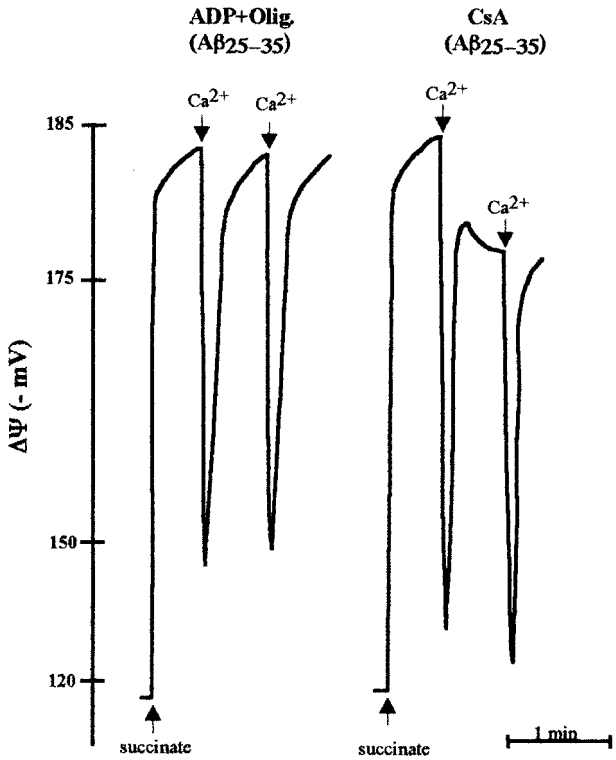

Fig. 3. Inhibitory effect of CsA and oligomycin plus ADP on $A\beta_{25-35}$-dependent permeability transition pore opening. Mitochondria were incubated at 0.8 mg protein/ml under standard conditions as described in Materials and Methods. 0.85 μM CsA and 1 mM ADP plus 2 μg/ml oligomycin were incubated with mitochondria for two minutes before adding $A\beta_{25-35}$. The two $A\beta_{25-35}$ (50 and 100 μM) were added five minutes before energizing the mitochondria with 5 mM succinate. The traces are typical of four or five experiments.

i.e., higher $A\beta_{25-35}$ (100 μM) (Fig. 5C) led to a faster PTP opening and, consequently to a smaller calcium retention within mitochondria. These effects on calcium accumulation could be prevented in the presence of 0.85 μM CsA and 1 mM ADP plus 2 μg/ml oligomycin. Although both agents contributed for a higher capacity of mitochondria to accumulate Ca^{2+}, the protection exerted by ADP plus oligomycin, as occurred on $\Delta\Psi m$, was more effective than that exerted by CsA (Fig. 5C, D).

DISCUSSION

A potentially central factor in cell death in neurodegeneration is the PTP. This channel has been shown to be involved in oxidant-induced mitochondrial large amplitude swelling (Bernardi *et al.*, 1994; Zoratti and Szabo, 1995), Ca^{2+} release

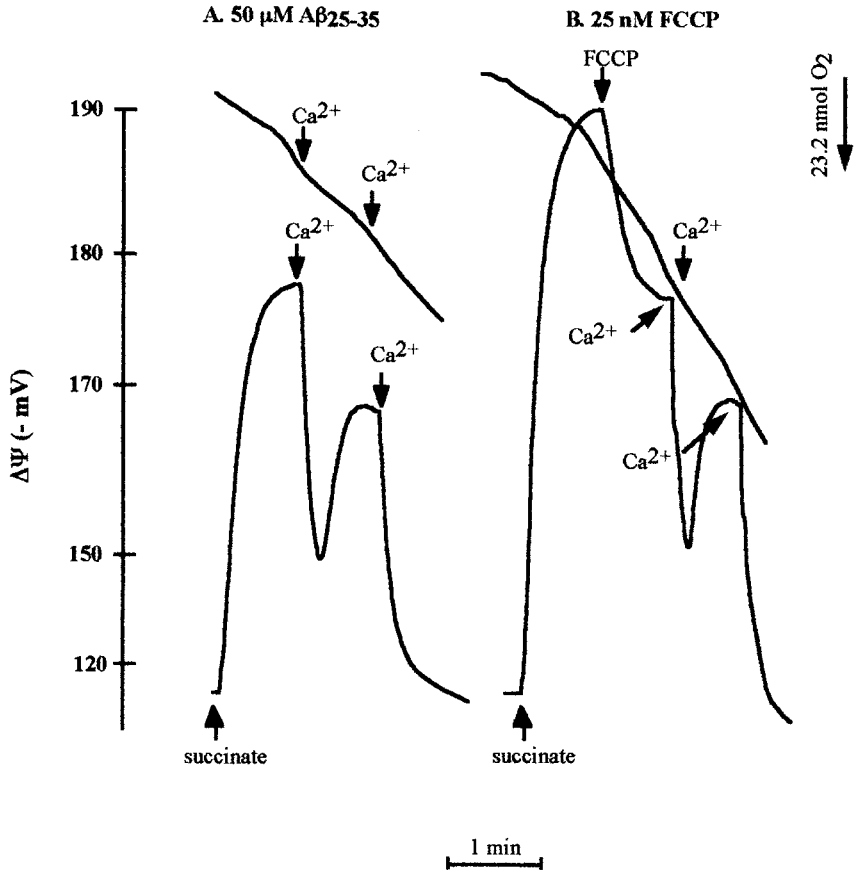

Fig. 4. Effect of $A\beta_{25-35}$ and FCCP on mitochondrial membrane potential and respiration. Freshly isolated brain mitochondria (0.8 mg) in 1 ml of the standard medium supplemented with 3 μM TPP^+ and 2 μM rotenone were energized with 5 mM succinate. A. 50 μM $A\beta_{25-35}$ was pre-incubated for five minutes at 30°C before mitochondria energization. B. 25 nM FCCP was added one minute after mitochondria energization. The traces are typical of two experiments.

(Bernardi *et al.*, 1994), and cell death (Marchetti *et al.*, 1996). Pore opening is controlled by membrane voltage and affected by numerous agonists and antagonists. Some of the effectors, which increase the PTP's opening probability, include Ca^{2+}, Pi (Bernardi and Petronilli, 1996) oxidizing agents (Bernardi *et al.*, 1994; Zoratti and Szabo, 1995), atractyloside, and inhibitors of the electron transport chain (Chernyak and Bernardi, 1996).

Several studies have demonstrated the existence of PTP in brain mitochondria (Andreyev and Fiskum, 1999; Kim *et al.*, 1999; Berman *et al.*, 2000; Brustovetsky and Dubinsky, 2000; Kristián *et al.*, 2000). Preliminary evidence from experiments with isolated brain mitochondria (Kristal and Dubinsky, 1997) and neuronal cultures (Dubinsky and Levi, 1998) suggests that neuronal mitochondria can undergo an increase in membrane permeability transition under pathological conditions.

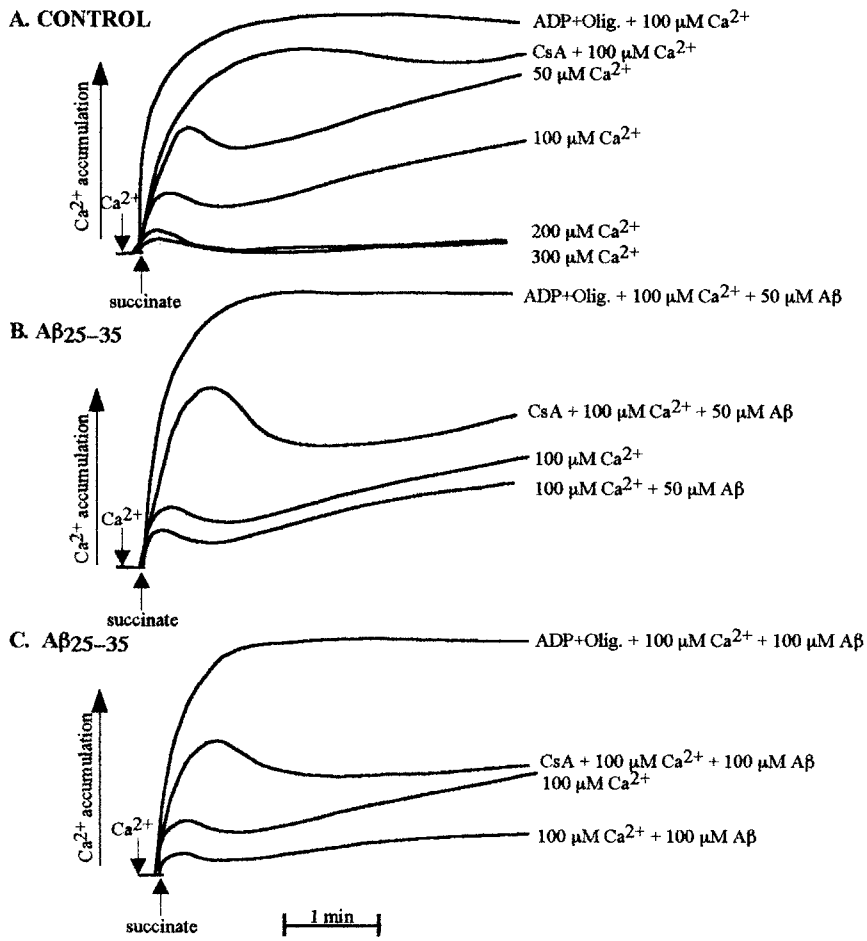

Fig. 5. Effect of $A\beta_{25-35}$ on calcium fluxes. Mitochondria were incubated at 0.8 mg protein/ml under standard conditions as described in Materials and Methods. A. Standard medium was supplemented with different Ca^{2+} concentrations (50, 100, 200, 300 μM) one minute before mitochondria energization with 5 mM succinate. B., C. 50 and 100 μM $A\beta_{25-35}$, respectively, were pre-incubated for five minutes before 100 μM Ca^{2+} addition. 0.85 μM CsA and 2 μg/ml oligomycin plus 1 mM ADP were added to the reaction medium two minutes prior to Ca^{2+} or $A\beta_{25-35}$ pre-incubation. The uptake and release of sequestered Ca^{2+} by mitochondria were monitored as described in Materials and Methods. The traces are typical of three experiments.

In this work, we demonstrate the involvement of Aβ on the promotion of brain PTP induced by Ca^{2+}. The mitochondrial $\Delta\Psi m$, mitochondrial respiration and calcium fluxes were the parameters studied since they are hallmarks of PTP. Mitochondrial depolarization favors PTP induction, presumably by lowering thresholds for calcium or oxidants (Bernardi, 1992). Extensive calcium cycling, the continual movement of calcium into mitochondria via the calcium uniporter and out of the mitochondria via the Na^+/Ca^{2+} exchanger, can also depolarize mitochondria and lead to a membrane permeability transition (Kristal and Dubinsky, 1997). Similarly

to other studies (Kristal and Dubinsky, 1997; Berman *et al.*, 2000; Kristián *et al.*, 2000) we observe PTP induction by extramitochondrial calcium addition. Ca^{2+} uptake is concentration-dependent, with higher concentrations leading to a lower amount of Ca^{2+} being accumulated by mitochondria, indicating a continuous cycling of Ca^{2+} due to PTP induction. However, there was always a retention of some amount of Ca^{2+} (Fig. 5) indicating that some mitochondria did not undergo PTP. Kristián *et al.* (2000) using succinate as substrate observed that, in brain mitochondria, following saturation of the Ca^{2+} uptake system, a large release of sequestered calcium did not ocur.

$A\beta_{25-35}$ decreased the $\Delta\Psi m$ and the capacity of mitochondria to accumulate Ca^{2+}, accelerating PTP induction (Fig. 1B, C; Fig. 5B, C). Parks *et al.* (2001) demonstrated that, in liver mitochondria, $A\beta_{25-35}$, in the presence of Ca^{2+} and phosphate, induces PTP in a concentration-dependent manner. These results are in agreement with other studies demonstrating that $A\beta$ peptides can cause a harmful elevation of intracellular calcium levels (Mattson *et al.*, 1992). We observe that PTP induction is potentiated in the presence of $A\beta$ suggesting that $A\beta$ establishes conditions promoting calcium cycling, and thus, may predispose mitochondria to PTP induction (Fig. 5B, C).

To elucidate between a direct or an indirect action of $A\beta_{25-35}$ on PTP, we compared the effect exerted by this peptide with that caused by the mitochondrial respiratory chain uncoupler FCCP. For this purpose, we used a small amount of FCCP (25 nmol/mg protein) in order to obtain a decrease in $\Delta\Psi$ similar that promoted by $A\beta_{25-35}$. Both agents, FCCP and $A\beta_{25-35}$, produced a similar effect: diminished $\Delta\Psi m$, the capacity of mitochondria to accumulate Ca^{2+} (Fig. 1B, C, 4A, B, 5B, C) and led to a complete uncoupling of the respiration (Fig. 4A, B). Uncoupling refers to loss of respiratory control, i.e., the proton gradient is no longer coupled to oxidative phosphorylation. Since uncouplers destroy the capacity for ATP synthesis, they are highly toxic compounds. There are numerous evidences (Mattson, 1997; Pereira *et al.*, 1999) indicating an imbalance on energetic metabolism induced by $A\beta$ peptides.

The reverse sequence of $A\beta_{25-35}$ ($A\beta_{35-25}$) did not induce the PTP (Fig. 2). This result allows us to conclude that a specific sequence of amino acids is needed for the induction of PTP. Mattson *et al.* (1993) stated that the "reverse sequence" $A\beta_{35-25}$ is not cytotoxic. Also, in a cell-based assay, $A\beta_{25-35}$ generated ROS whereas $A\beta_{35-25}$ is not cytotoxic. Furthermore, $A\beta_{25-35}$ was able to initiate synaptosomal lipoperoxidation, whereas $A\beta_{35-25}$ had no effect (Butterfield *et al.*, 1994).

In Fig. 5, we observed the accumulation–release–recuperation behavior of brain mitochondria probably due to the heterogeneity of brain mitochondria population. A more susceptible mitochondrial sub-population to toxic agents undergoes PTP and releases Ca^{2+} which can be reuptaken by other mitochondria. The $\Delta\Psi m$ data (Fig. 1) also confirmed this heterogeneity: after the first Ca^{2+} challenge, a small drop on $\Delta\Psi m$ occurred, this drop being probably due to the injury of the mitochondrial sub-population more susceptible to insults. This heterogeneity of brain mitochondria population was previously reported (Berman *et al.*, 2000; Kristián *et al.*, 2000).

After $A\beta_{25-35}$ and/or Ca^{2+} induced depolarization, EGTA (a Ca^{2+}-chelating agent) and ADP (an inhibitor of PTP) plus oligomycin (prevents ADP phosphorylation) completely repolarized mitochondria (Fig. 1), an effect that has also been

demonstrated by others (Hunter and Haworth, 1979; Zoratti and Szabo, 1995; Brustovetsky and Dubinsky, 2000).

Another proof that $A\beta_{25-35}$ promotes PTP induction is given by the protection exerted by the immunosuppressant peptide and specific inhibitor of PTP, CsA. The effect of CsA on the ability of brain mitochondria to accumulate Ca^{2+} was only moderate when compared with the effects described for CsA on liver or muscle mitochondria (Fontaine *et al*., 1998; Kristián *et al*., 2000). Similar effects of CsA were also reported on cell lines (Murphy *et al*., 1996) and neuronal primary cultures (Dubinsky and Levi, 1998). Although CsA does not give a total protection against PTP induction, it increases the capacity of brain mitochondria to accumulate Ca^{2+} (Figs. 3, 5). However, when ADP plus oligomycin were present initially, the $A\beta_{25-35}$ and/or Ca^{2+}-induced depolarization was prevented. Brustovetsky and Dubinsky (2000) also reported the protection exert by ADP plus oligomycin on brain mitochondria before calcium challenge.

Our data clearly shows that the specific amino acid sequence $A\beta_{25-35}$, affects directly mitochondria leading to the uncoupling of respiration and, consequently, to promotion of PTP, thus contributing to neuronal death. The understanding of the mechanisms underlying the neurogenerative process induced by Aβ peptide is a fundamental step for the finding of new therapeutical strategies in Alzheimer's disease.

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