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Cyclosporin A targets involved in protection against glutamate excitotoxicity

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Abstract

The toxicity of glutamate in neuronal cultures has been attributed in part to a mitochondrial dysfunction involving the permeability transition pore. The participation of the permeability transition pore in this process has been pharmacologically demonstrated by the use of cyclosporin A, which inhibits pore opening by interaction with mitochondrial cyclophilin and, thus, prevents cell death and upstream events. Since cyclosporin A also acts on calcineurin, we have investigated which of the targets of cyclosporin A was responsible for the inhibition of glutamate-excitotoxicity in cerebrocortical primary neuronal cultures. Reactive oxygen species production and early (30 min to 2 h) drop in ATP levels are initial events in glutamate excitotoxicity taking place before neuronal death. Cyclosporin A did not inhibit reactive oxygen species production, but reduced the drop in ATP levels and subsequent neuronal death. However, cyclosporin derivatives that do not bind to calcineurin had smaller effect on survival than cyclosporin A, (regardless of whether they were able to bind cyclophilin), indicating that cyclosporin A protects against glutamate toxicity also through calcineurin-related mechanisms. Consistent with this view, ATP loss appears to result from nitric oxide synthase (NOS) activation (including calcineurin-dependent dephosphorylation) and nitric oxide (NO)/peroxinitrite-dependent increase in poly (ADP-ribose) polymerase activity, since it was reduced by inhibitors of these activities. Collectively, these results suggest that cyclosporin A exerts its protective effects through calcineurin-dependent and independent mechanisms.

Keywords: Cerebrocortical neuronal culture; Glutamate excitotoxicity; Mitochondrion; Permeability transition pore; Reactive oxygen species; Cyclophilin; Cyclosporin A; Calcineurin; Nitric oxide (NO) synthase; Adenine nucleotide; Poly (ADP-ribose) polymerase

1. Introduction

Glutamate is the main excitatory neurotransmitter in the central nervous system. As such, it plays a crucial role in synaptic transmission in the brain. However, glutamate also behaves as a double edged sword since the persistent activation of glutamate receptors, specifically of the *N*-methyl-D-aspartate (NMDA) type, has deleterious effects on neuronal survival, for instance, after ischemia-induced neuronal death (Chihab et al., 1998). The mechanism of neuronal death triggered by glutamate is believed to be typically necrotic (Choi, 1996), but glutamate may induce delayed apoptosis (Ankarcrona et al., 1995; Portera-Cailliau et al., 1997). Despite the paradigmatic opposition between apoptosis and necrosis, these two modes of death may share common steps, particularly with regards to the involvement of mitochondria (Zamzami et al., 1997).

In ischemia, the participation of mitochondria may involve recruiting the mitochondrial permeability transition pore (Griffiths and Halestrap, 1993, Uchino et al., 1995), a pore that allows the free diffusion of ions and small molecules and, consequently, a collapse of $\Delta\Psi_{mit}$ (Bernardi and Petronilli, 1996; Zamzami et al., 1997). Hypoglycemic damage to neurons also involves the mitochondrial permeability transition pore (Friberg et al., 1998; Khaspekov et al., 1999). Mitochondria plays also a role in glutamate-induced neuronal death (Ankarcrona et al., 1995, 1996; Isaev et al., 1996; Nieminen et al., 1996; Schinder et al., 1996;

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Stout et al., 1998). Indeed, preincubation with mitochondrial inhibitors together with oligomycin (to prevent ATP rundown) protects against glutamate-induced necrotic death and Ca^{2+} deregulation in cerebellar granule cells (Nicholls and Budd, 1998) or NMDA-induced toxicity in rat hippocampal neurons (Sengpiel et al., 1998), and mitochondrial uncouplers protect rat forebrain neurons from glutamate toxicity (Stout et al., 1998). As with ischemia, it has been suggested that mitochondria participation in glutamate excitotoxicity involves permeability transition pore opening (Nieminen et al., 1996; Schinder et al., 1996). However, the drop in $\Delta\Psi_{mit}$ observed during glutamate-induced neuronal death, which has been taken as the signature of the permeability transition, is also part of the physiological response of mitochondria to NMDA receptor activation-induced Ca^{2+} accumulation (Duchen, 1992, White and Reynolds, 1995, Peng and Greenamyre, 1998, Kiedrowski, 1999). Therefore, permeability transition pore recruiting, which leads to a drop in $\Delta\Psi_{mit}$ in many other settings, cannot be unambiguously proposed as a mechanism to explain the role of mitochondria in glutamate toxicity (see also Bernardi et al., 1999). Present evidence indicating that the fall in $\Delta\Psi_{mit}$ during glutamate excitotoxicity involves opening of mitochondrial permeability transition pore, relies in the use of cyclosporin A as a pore blocker (Ankarcrona et al., 1996; Isaev et al., 1996; Nieminen et al., 1996; Schinder et al., 1996; White and Reynolds, 1996).

Cyclosporin A is thought to block permeability transition pore opening by interacting with mitochondrial cyclophilin (Bernardi and Petronilli, 1996; Zamzami et al., 1997). However, cyclosporin A is an established drug used as immunosuppressant by virtue of its ability to inhibit calcineurin and, thus, prevent the dephosphorylation of the T-cell specific transcription factor, nuclear factor of activated T cells (NF-AT), and thereby block interleukin 2 (IL-2) transcription (Liu, 1993). The immunosuppressive action of cyclosporin A requires the formation of binary complex of cyclosporin with a member of the cyclophilin family of proteins. Cyclophilins have peptidyl-prolyl isomerase (rotamase) activity, which is inhibited by cyclosporin A; however, this activity is not involved in immunosuppression (Liu, 1993). The composite cyclosporin A/cyclophilin surface binds to calcineurin and inhibits its phosphatase activity (Papageorgiou et al., 1994). Dawson et al. (1993) showed that calcineurin inhibitors cyclosporin A and tacrolimus (FK506) prevented glutamate neurotoxicity through their effect on calcineurin. Therefore, the question of whether cyclosporin A blocks neuronal death by acting exclusively on mitochondrial cyclophilin and the permeability transition pore remains to be established.

The purpose of this study was twofold: (i) to identify other early events in glutamate excitotoxicity that may be markers of an irreversible pore opening and (ii) to study whether the mitochondrial permeability transition pore is involved in glutamate excitotoxicity with the use of specific inhibitors derived from cyclosporin A.

2. Materials and methods

2.1. Primary neuronal culture

Cerebral cortex neuronal cultures were prepared from 18-day-old Wistar rat embryos as described in Villalba et al. (1994) and Ruiz et al. (1998). Culture medium was based in a serum-free defined medium formulation supplemented with different concentrations of heat-inactivated horse serum (Ruiz et al., 1998). The dissociated cell suspension was plated at $1–1.5 \times 10^5$ cells/cm^2 on poly-L-lysine and laminin pretreated plastic or glass coverslips. Cells were plated in defined medium containing 20% horse serum for 2–3 h to let the cells attach to the surface, and then cultured in reduced serum concentration for 2 days (5% horse serum in defined medium). After this initial period, cultures were switched to serum-free defined medium, two thirds of which was replaced every second day. The different cell types in the cultures were characterized with specific antibodies (Ruiz et al., 1998). Under the present culture conditions, neurons represented $81.8 \pm 4.3\%$ of the cell population, astrocytes were $8.6 \pm 0.8\%$, and occasional microglial cells were detected ($0.8 \pm 0.3\%$,). 8.7% of the cells did not fall into any of these groups.

2.2. Neuronal viability assays

After 7 days in vitro, the culture medium was replaced by pyruvate-free defined medium. Then, 2–4 days later (at 9–11 days in vitro), neurons were exposed to glutamate, or different excitotoxic agents (glutamate, NMDA, or as indicated) or vehicle alone (in pyruvate-free minimal essential medium with Earle's salts (GIBCO), P-MEM) (Ruiz et al., 1998). After a 30-min incubation, cultures were rinsed with P-MEM and incubated in pyruvate-free defined medium at 37°C in 7% CO_2. Cell death was determined by measuring released lactate dehydrogenase (LDH) by damaged cells to the medium and by calcein/propidium iodide (PI) uptake (Ruiz et al., 1998).

In order to study the caspase dependence of glutamate-induced neuronal death, we used two inhibitors of caspase-1 and group I caspases both at 100 μM: Acetyl-tyrosyl-valyl-alanyl-aspartinal, Ac-YVAD-CHO, and Ac-Tyr-Val-Ala-Asp-chloromethylketone, Ac-YVAD-CMK (Garcia-Calvo et al., 1998). Ac-leu-leu-argininal (leupeptin) and Tos-lys-chloromethylketone · HCl were used as control peptides, respectively. All peptides were from Peninsula. Cultures were preincubated with the inhibitors or control peptides 1 h prior to glutamate exposure and maintained during and after (24–72 h) the addition of glutamate.

2.3. Mitochondrial membrane potential

The relative variations in $\Delta\Psi_{mit}$ were determined with rhodamine 123. Neurons ($250–300 \times 10^3$ cells/well) were incubated with rhodamine 123, added to the culture medium (1 μg/ml) in the presence or absence of 2-μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP)/2-μM oligomycin (to depolarize mitochondria while preventing the rundown of ATP by mitochondrial ATPase), for 10 min at 37°C. Then the medium was aspirated and cells were washed twice with 136-mM NaCl, 2.68-mM KCl, 9.46-mM sodium phosphate, pH 7.4, (phosphate buffered saline) at room temperature. Rhodamine 123 was extracted from the cells with 2 ml butanol, and its fluorescence was determined in an Aminco Bowman Series 2 spectrofluorometer (excitation, 485 nm; emission, 532 nm). The main effects of FCCP are mitochondrial depolarization and, therefore, FCCP-releaseable rhodamine 123 (difference between rhodamine 123 uptake in the absence and presence of FCCP/oligomycin expressed as percentage of total rhodamine 123 uptake) may be used to estimate $\Delta\Psi_{mit}$. However, since FCCP may also partly depolarize the plasma membrane as a result of equilibration of the proton gradient (Martínez-Serrano and Satrústegui, 1992) and, thus, not accurately correct for nonmitochondrial rhodamine 123 accumulation, the estimation of $\Delta\Psi_{mit}$ is only a relative one.

2.4. Adenine nucleotide levels

Neurons ($2.4–3.6 \times 10^6$) were mechanically detached, centrifuged (1500 rpm, 5 min), and adenine nucleotides were extracted from the pellets with 0.050–0.075 ml of 100-mM Tris, 4-mM EDTA, 1% trichloroacetic acid for 20 min in ice. Extracts were collected (8000 rpm, 1 min, Eppendorf table centrifuge) and supernatants assayed immediately.

Adenine nucleotides (ATP and ADP) were assayed in a final volume of 1 ml in 100-mM Tris, 4-mM EDTA, 10-mM $MgCl_2$, luciferase and luciferin (ATP bioluminescence CLS, Boehringer Mannheim) following the supplier's instructions. Chemioluminescence was recorded in an LKB 1250 luminometer. ATP was evaluated from the bioluminescence signal obtained after addition of the extracts (0.03 ml), which was calibrated with appropriate ATP standards. After the ATP signal was obtained, ADP present in the extracts was evaluated after conversion to ATP by adding 2.5-mM fosfocreatine and 4 units of creatine kinase. The increase in luminescence obtained under these conditions was calibrated with appropriate ADP standards.

2.5. Hydroethidine-based determination of oxygen-derived free radicals

A hydroethidine stock (dihydroethidium from Molecular Probes) was prepared at 10 μg/μl in dry dimethylsulfoxide as indicated by Bindokas et al. (1996). Assays were conducted on the stage of an upright Zeiss LSM confocal microscope. Neurons growing on coated glass coverslips were transferred to the observation chamber (37°C), and incubated with 1–3-μM hydroethidine (in P-MEM with Earle's salts (GIBCO), 10-mM HEPES). Acquisition of ethidium fluorescence images was started immediately and continued for about 30 min. Confocal images were collected using a water immersion 40 ×, Achroplan, 0.75 NA objective. A pinhole of 100 was used with an excitation filter of 514 nm (100 × attenuation) and an emission filter of > 590 nm. Images (512 × 512 pixels) were obtained by averaging 16–32 scans. Settings (contrast, brightness, pinhole, zoom) used during image acquisition were maintained within a same experiment and in parallel experiments. Illumination was limited to periods of image acquisition.

To expose neurons to glutamate (100–500 μM), this was added as a bolus (1–5 mM in 1/10 of the chamber volume) after one or two images were acquired (around 3–5 min after the addition of hydroethidine) and when the average fluorescence intensity of the field was very small and the cell outlines were not apparent. Images were acquired every 2–4 min and processed using the LSM 3 Laser Scan Microscope software, which allows to measure the average fluorescence intensity within individual neurons. These were digitally traced from the images obtained at the end of the experiment when the production of ethidium from hydroethidine was sufficient to visualize the outlines of the cell bodies.

With this procedure, prior to glutamate addition, there was no fluorescence in the cells except that corresponding to a few damaged neurons and this did not change significantly during the course of the experiment in vehicle-treated cultures. Castilho et al. (1999) have argued that when high hydroethidine concentrations are used (5 μM and above) the apparent increase of fluorescence produced by mitochondrial uncouplers is not due to an increase in mitochondrial O_2^- production. Rather, it would arise from a depolarization-dependent release of ethidium that had been previously accumulated in mitochondria with a corresponding increase in fluorescence yield. However, our working conditions (no preincubation with hydroethidine) preclude any accumulation of the dye in mitochondria before glutamate addition, and suggest that the increase in fluorescence really reports an increase in O_2^- formation.

3. Results

3.1. Time course of glutamate-induced neuronal death

Glutamate-induced neuronal death in our rat cortical cultures is mainly due to NMDA receptor activation (Ruiz et al., 1998). To study the time course of glutamate-in-

duced neuronal death in cortical cultures, neuronal viability was assessed by calcein/PI uptake (that reports live and dead cells, respectively) and by measuring LDH release. The addition of 100-μM glutamate induced an abrupt increase in the percentage of dead cells measured by calcein/PI uptake that reached about 40% after 6 h (Fig. 1E). Cell death remained relatively constant from then onwards. Dead cells had swollen nuclei (Fig. 1D), one of the signatures of necrotic cell death (Ankarcrona et al., 1995). The cells surviving glutamate exposure included mainly astrocytes, as judged by glial fibrillary acidic protein immunoreactivity (results not shown). When evaluated by LDH release, neuronal death was lower, as described previously (Ruiz et al., 1998), amounting to around 13% at 24 h. However, the initial rapid phase of neuronal death taking place between 2 and 6 h after glutamate addition is also clearly observed (Fig. 1E).

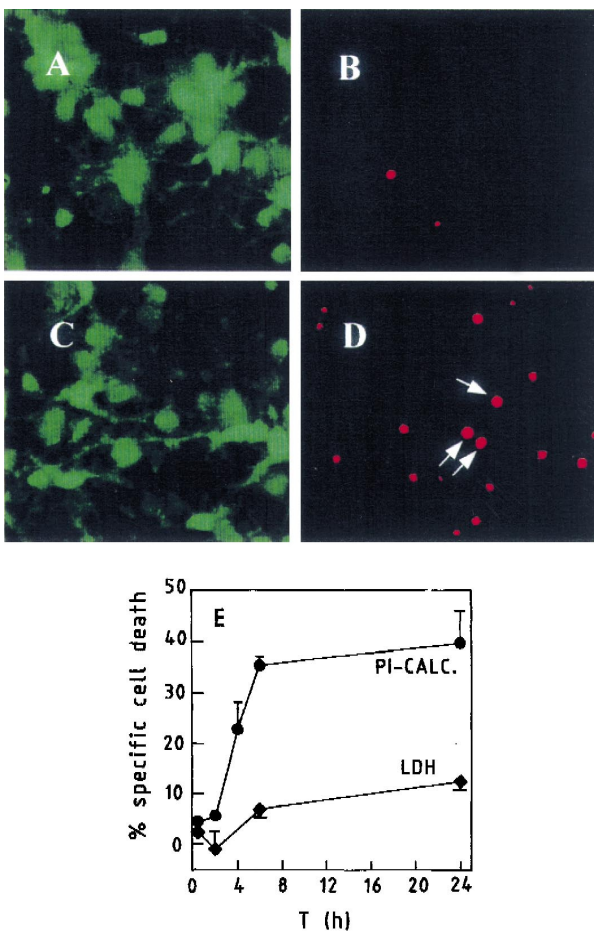

Fig. 1. Time course of glutamate-induced neuronal death in cerebral cortex cultures. Sets of sister cortical cultures were exposed to 100-μM glutamate or vehicle for 30 min and neuronal death was evaluated at different times by calcein/PI staining or LDH release. (A, B, C, D) Neuronal culture doubly stained with calcein (green) and PI (red) at 6 h after vehicle (upper panels) or glutamate (lower panels) addition. Swollen nuclei are indicated by arrows. (E) The results reflect specific glutamate-induced neuronal death at different times after glutamate or vehicle addition, i.e, the percentage of dead cells (calcein/PI) or LDH release in glutamate-exposed cultures minus the nonspecific death or LDH release obtained in vehicle-treated cultures. The statistical significance of the difference between cell death obtained with glutamate or vehicle was $P < 0.05$; (at 4 and 24 h) or $P < 0.0005$ (at 6 h), when death was evaluated by calcein/PI, and $P < 0.0005$ for 6 and 24 h, when death was evaluated by LDH release (ANOVA, post hoc Bonferroni test).

NMDA-induced death of mixed cerebrocortical neurons in culture appears to involve necrosis or apoptosis depending on whether NMDA concentrations are high (2 mM) or low (300 μM), respectively (Tenneti et al., 1998). In our culture conditions, where the percentage of glial cells (8.6%, see Section 2) is probably much smaller than in that of Tenneti et al. (1998), a low glutamate concentration (100 μM) elicited what appears to be a full necrotic response in about 6 h, even though manipulations to increase NMDA receptor activation (omission of Mg^{2+} or presence of glycine, (Ankarcrona et al., 1995; Tenneti et al., 1998) were avoided. The response consisted in a rapid cell death reflected in (i) the simultaneous loss of calcein uptake and appearance of PI-stained, swollen nuclei, and (ii) a parallel release of LDH. This rapid necrosis was unaffected by the presence of the caspase inhibitors Ac-YVAD-CHO and Ac-YVAD-CMK (results not shown). Glutamate also elicited a delayed neuronal death in our cerebrocortical cultures at 48–72 h after glutamate challenge, which was variable and much smaller in magnitude than the initial necrosis. This delayed process was probably apoptotic since at these late times, the caspase inhibitors had a small protective effect (10%, results not shown), and has not been studied any further.

3.2. Effects of glutamate on mitochondrial polarization, adenine nucleotide levels and O_2^- production

Glutamate addition to primary cortical neuronal cultures results in a rapid increase in cytosolic Ca^{2+}, which is maintained at elevated levels long after glutamate withdrawal (Ruiz et al., 1998). At the same time, i.e., within the first 10 min after glutamate addition, there is a rapid decrease in FCCP-releasable rhodamine 123, which is partially reversed 1.5 h later (Fig. 2A). A new decrease is observed at 3.5 h, which is not reversed thereafter. These two phases of rhodamine 123 loss closely match the mitochondrial depolarization events reported by Schinder et al. (1996) and Vergun et al. (1999). We have verified that the initial (10 min) decrease in FCCP-releasable rhodamine 123 is due to changes in mitochondrial membrane potential and not to plasma membrane depolarization, by exposing neurons to high K^+ (50-mM KCl) in a Ca^{2+}-free medium (Ca^{2+}-free defined medium, 50-μM ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA)). The acute neuronal response to high K^+ in calcium-free medium is plasma membrane (but not mitochondria) depolarization (Duchen, 1992). Similarly, no variation in FCCP-releasable rhodamine 123 was observed during the first 10 min of high K^+ exposure ($27.2 \pm 3.4\%$ in 50-mM KCl, and $30.1 \pm 1.6\%$ in control conditions,

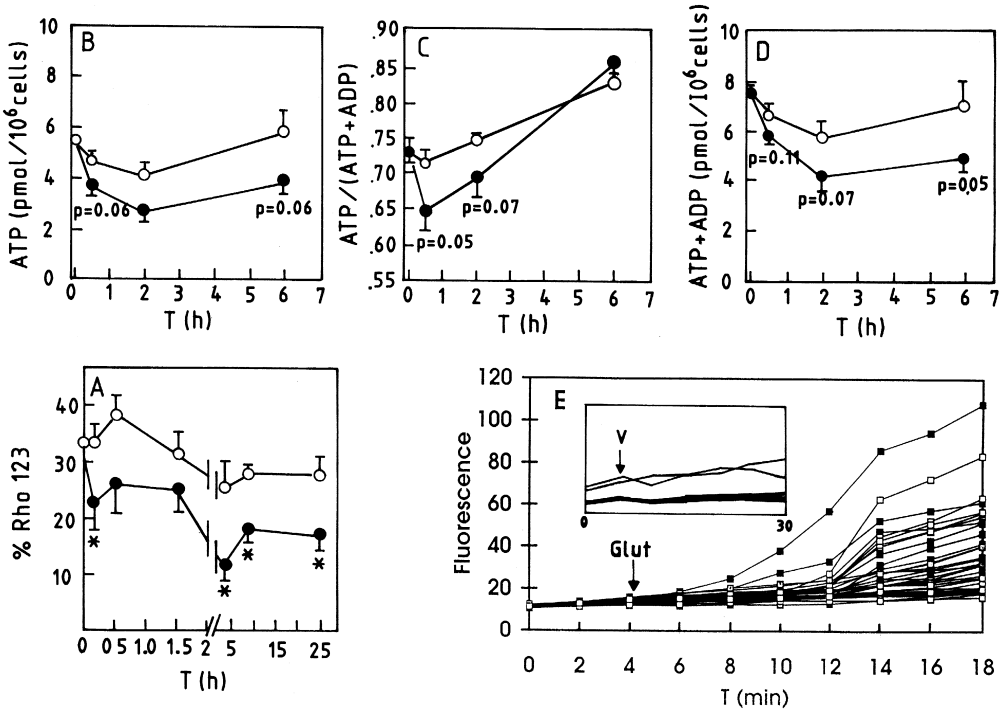

Fig. 2. Changes in FCCP-releaseable rhodamine 123 accumulation, adenine nucleotide levels and hydroethidine oxidation after glutamate stimulation. A. Cerebrocortical cultures were exposed to glutamate (100 μM, closed circles) which was added at time 0 and maintained for 30 min, and rhodamine 123 (Rho 123) accumulation was assayed at the times indicated. Rhodamine 123 accumulation in vehicle-exposed cells (open circles) is also shown. The results reflect FCCP/oligomycin releaseable rhodamine 123 (i.e., difference between Rhodamine 123 uptake in the absence or presence of FCCP/oligomycin), expressed as percentage of total rhodamine 123 taken up by the cells, and represent means ± S.E.M. of 3–11 experiments performed in triplicate. Difference with respect to vehicle-exposed neurons: $^{*}P < 0.05$, unpaired *t*-test. (B, C, D) Glutamate was added at time 0 and neurons were switched to a glutamate-free medium 30 min later. Adenine nucleotides were extracted at the times indicated. Changes in ATP levels (B), the ATP/(ATP + ADP) ratio (C) or ATP + ADP levels (D) in glutamate- (closed circles) or vehicle-treated neurons (open circles) are shown. Values were expressed as a function of the total number of living cells at the different time points, based on the percentage of LDH release and represent means ± S.E.M. of 3–11 experiments performed in triplicate. The significance of the difference between glutamate- and vehicle-treated cells is indicated. $^{*}P < 0.05$ two-tailed, unpaired *t*-test. E. Cortical neurons were incubated with 3-μM hydroethidine on the stage of the confocal microscope during the times indicated. At time 2 min, glutamate (100 μM) was added. Ethidium fluorescence was evaluated in every cell in the field; each tracing corresponds to an individual cell. The results illustrate a representative experiment. The insert shows the results of a control experiment, where vehicle (V) was added as indicated. The two upper traces are from damaged neurons and the lower broad band corresponds to about 25 overlapping traces from individual neurons. The fluorescence scale is the same as panel E but notice the difference in time scale.

respectively), indicating that the initial drop observed in Fig. 2A reflects mainly mitochondrial depolarization.

If mitochondria are persistently depolarized due to permeability transition pore opening, ATP production should cease as long as the pore is open, and, together with hydrolysis by the mitochondrial ATPase, this would lead to ATP depletion (Bernardi et al., 1999). Since it is known that glutamate neurotoxicity involves a fall in ATP levels (Ankarcrona et al., 1995; Budd and Nicholls, 1996), we have studied the changes in adenine nucleotide levels as a possible marker of pore opening. We have found that high energy nucleotides and the ATP/(ATP + ADP) ratio decreased in cortical neuronal cultures shortly after glutamate addition (Fig. 2B–D). Changes in the same direction, albeit of much smaller magnitude, also took place in vehicle-exposed cultures (Fig. 2), probably because the manipulations (medium changes, rinses) of the 30-min exposure to vehicle represent a somewhat stressful condition to neurons. ATP and ATP + ADP levels in glutamate-exposed cells decreased at 30 min, (20% and 15%, respectively), and further decreased at 2 h (34% and 31%, respectively) closely matching the changes in adenine nucleotide concentration in transient focal ischemia in vivo (Folbergrova et al., 1995). The decrease in ATP, ATP + ADP and ATP/(ATP + ADP) preceded cell death, which was only significant after 4 h of glutamate exposure (Fig. 1). Moreover, 6 h after glutamate addition, when the process of glutamate-induced neuronal death was largely completed (Fig. 1), the ATP/(ATP + ADP) ratio returned to control levels (Fig. 2C). This indicates that the neurons that survive glutamate excitotoxicity have normal levels of adenine nucleotides, a result consistent with those of Ankarcrona et al. (1995) in cerebellar granule neurons.

Glutamate addition to neurons leads to an increased production of reactive oxygen species (Reynolds and Hastings, 1995; Bindokas et al., 1996; Castilho et al., 1999). A burst of reactive oxygen species production is induced after permeability transition pore opening in different cell

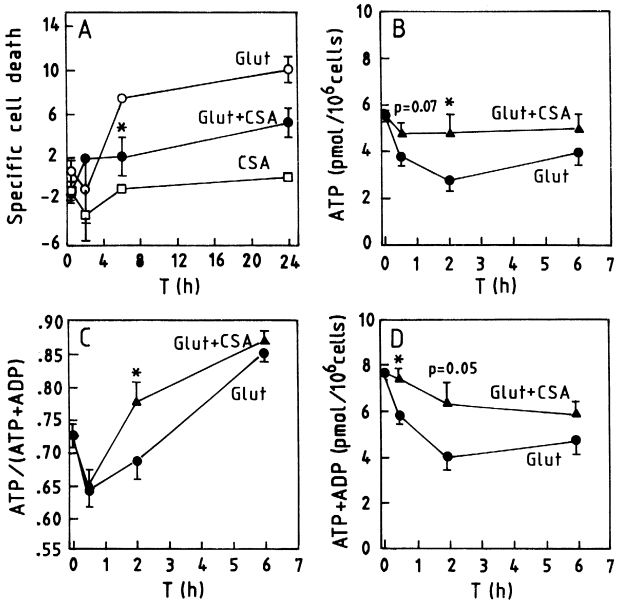

Fig. 3. Effect of cyclosporin A on the glutamate-induced fall of ATP and LDH release. 100-μM glutamate was added at time 0 and washed off after 30 min. LDH release (A) or adenine nucleotides (B, C, D) were measured at the times indicated cyclosporin A (5 μM) was present (closed circles) or absent (open circles) 30 min before glutamate addition and maintained onwards. (A) Time course of LDH release after glutamate addition in the absence (open circles) or presence (closed circles) of cyclosporin A. Specific LDH release was obtained after subtracting that released in vehicle-exposed cultures with or without cyclosporin A. The effect of cyclosporin A itself is represented by open squares. Results are means ± S.E.M. of three experiments. Significance of the difference in the presence or absence of cyclosporin A: $^{*}P < 0.05$; unpaired *t*-test. (B, C, D) Adenine nucleotide levels in cultures exposed to glutamate in the absence (closed circles) or presence (closed triangles) of cyclosporin A. Time course of the changes in ATP levels (B), ATP/(ATP + ADP) ratio (C), and normalized ATP + ADP values, with respect to control, vehicle-exposed neurons (D). The presence of cyclosporin A by itself did not modify adenine nucleotide levels in vehicle-exposed cultures. In all cases, the results were corrected for cell death at the different time points, estimated from LDH release. *P* values were $^{*}P < 0.05$ or as indicated (two-tailed, unpaired *t*-test).

types (Zamzami et al., 1997) and cerebral mitochondria produce reactive oxygen species when exposed to pore opening conditions, such as elevated Ca^{2+} plus Na^{+} (Dykens, 1994). Therefore, we have also studied reactive oxygen species production as a possible marker of permeability transition pore opening. We have used O_2^--sensitive and pH-insensitive hydroethidine (Bindokas et al., 1996) and studied its oxidation to ethidium in neurons after glutamate exposure by confocal imaging. Fig. 2E shows that an increase in fluorescence is detected about 14 min after glutamate addition, steadily increasing thereafter. This did not happen in control, vehicle-exposed cultures (see insert in Fig. 2E). The early O_2^- production occurs within the same time window as the membrane potential and ATP changes, and could also be a marker of permeability transition pore opening.

3.3. Effect of cyclosporin A and derivatives on glutamate-induced fall in adenine nucleotide levels and neuronal death

Fig. 3 shows the effects of 5-μM cyclosporin A, a potent blocker of the permeability transition pore through its interaction with mitochondrial cyclophilin (Petronilli et al., 1994; Nicolli et al., 1996), on the fall in adenine nucleotides produced by glutamate exposure. The transient fall in the ATP/(ATP + ADP) ratio observed at 30 min after glutamate addition is still present while control levels are rapidly recovered as soon as 2 h after glutamate addition (Fig. 3C). However, the fall in ATP (or ATP + ADP) at 30 min and 2 h is substantially reduced (Fig. 3B). Interestingly, at these same concentrations, cyclosporin A protects cortical neurons against glutamate-induced neuronal death, with a very pronounced effect on the initial necrosis occurring at 6 h (about 70% protection, Fig. 3A).

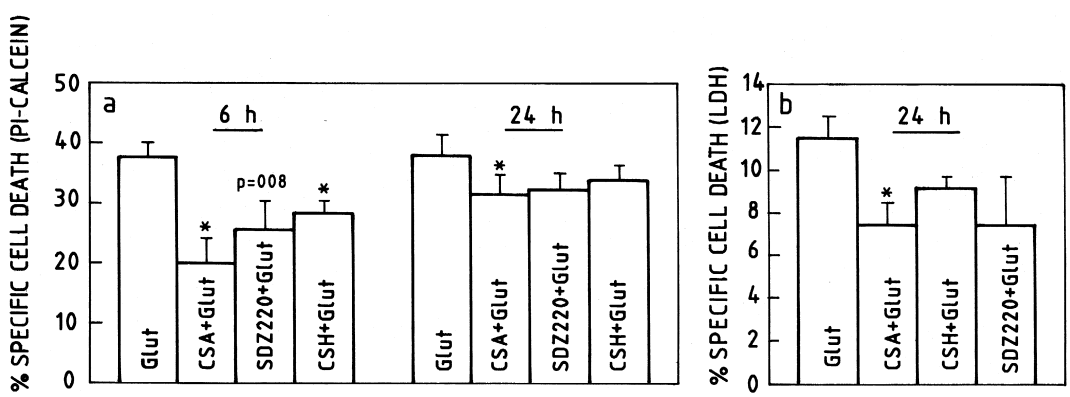

Fig. 4. Effect of cyclosporin A and derivatives on glutamate-induced neuronal death. Cyclosporin A, D-MeVal-11-cyclosporin (CSH), and MeVal-4-cyclosporin (SDZ220) were present at a concentration of 5 μM. Cortical neurons were preincubated with these compounds for 30 min before glutamate exposure and maintained during glutamate treatment and thereafter. Cell death was determined by the calcein/PI method or by LDH release, at 6 and 24 h after glutamate addition. Specific cell death was obtained by correcting for nonspecific cell loss in vehicle-exposed cultures (both for calcein/PI or LDH release), which was not affected by the different cyclosporin A derivatives used. Results are means ± S.E.M. of three independent experiments each performed in triplicate. *P* values correspond to the differences between incubations with glutamate alone or in the presence of the cyclosporin A derivatives ($^{*}P < 0.05$, paired *t*-test, two-tailed).

However, glutamate-induced O_2^- production was not inhibited in the presence of cyclosporin A (results not shown).

Protection by cyclosporin A is thought to occur via its interaction with mitochondrial cyclophilin, but it may also be due to its inhibitory effects on calcineurin. Cyclosporin A is a cyclic undecapeptide, which has 7 of the 11 amides in the *N*-methylated form. Only residues 9, 10, 11, 1, 2 and 3 of cyclosporin A are in contact with cyclophilin (Kd 10–100 nM, Papageorgiou et al., 1994; Kiessig et al., 1999). The remaining residues (4–8) protrude out the cyclophilin surface. This hydrophobic protrusion of cyclosporin A is termed the effector loop and makes specific interactions with calcineurin (Liu, 1993).

Even small chemical changes in residues of the effector loop, particularly at position 4 (MeLeu in cyclosporin A), can destroy the immunosuppressive effect without reducing the ability to bind cyclophilin A (Papageorgiou et al., 1994). This is why MeVal-4-cyclosporin (220–384) and other cyclosporin derivatives with side-chain modifications at position 4 do not inhibit calcineurin (110–2500 times less immunosuppressive activity), while affinity constants to cyclophilin A are unchanged (Husi and Zurini, 1994; Papageorgiou et al., 1994; Kallen et al., 1998). On the other hand, modifications in the side-chain residue at position 11 (MeVal in cyclosporin A) prevent both the binding to cyclophilins and inhibition of calcineurin. D-MeVal-11-cyclosporin does not bind to cyclophilin A (about 1000 lower affinity than cyclosporin A) and does not form complexes with calcineurin (Ryeffel et al., 1993; Husi and Zurini, 1994; Kiessig et al., 1999). Thus, to tell apart the possible effectors of cyclosporin A we have used two cyclosporin derivatives devoid of effects on calcineurin, D-MeVal-11-cyclosporin, which does not bind to cyclophilin A, and MeVal-4-cyclosporin, which does with the same affinity as cyclosporin A. Nicolli et al. (1996) have found that cyclophilin A and mitochondrial cy-

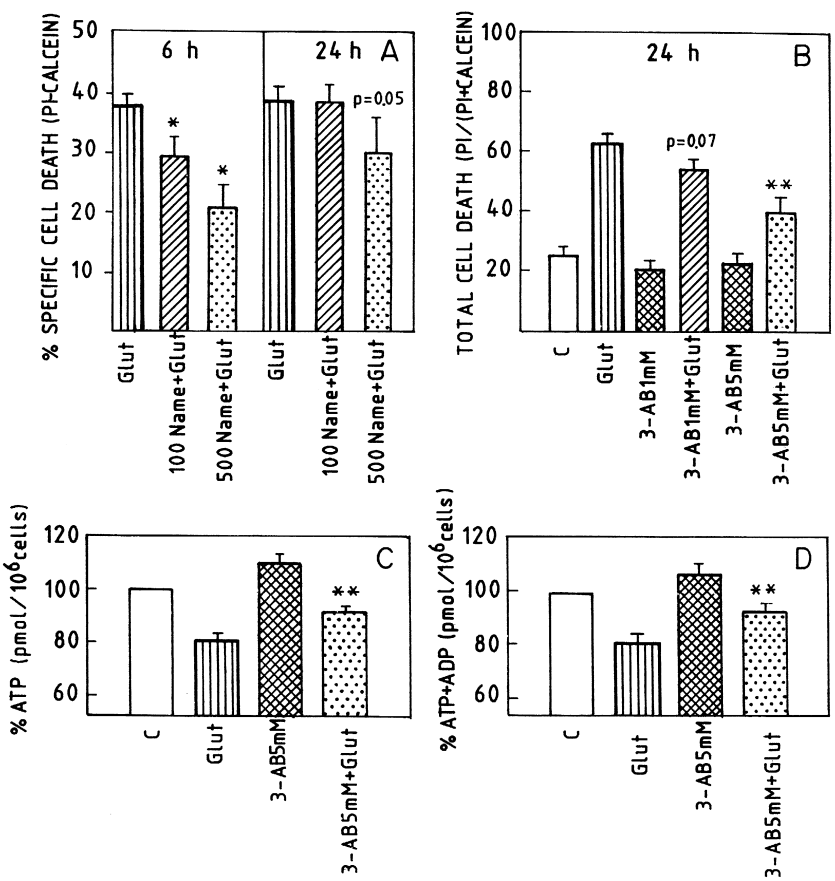

Fig. 5. Effect of NOS and Poly (ADP-ribose) polymerase inhibitors on glutamate-induced cell death and early drop in adenine nucleotides. Sets of sister cultures were preincubated with 100–500-μM L-NAME or with 1-mM or 5-mM 3-Aminobenzamide (3-AB) 30 min before glutamate or vehicle addition, and maintained during and after glutamate exposure. Neuronal cell death was estimated at 6 and 24 h after glutamate addition by calcein/PI staining (panel B). Values were corrected for cell death obtained in vehicle-treated cultures, to obtain specific cell death (panel A). Results correspond to means ± S.E.M. of three to four experiments. The difference between glutamate-induced cell death in the absence or presence of inhibitors was: $^{*}P < 0.05$ (two-tailed paired (A) or unpaired (B) *t*-test). (C and D) ATP and ADP were determined in these cultures 2 h after glutamate exposure. The results were corrected for the percentage of cell loss at 2 h as determined by LDH release, and are means ± S.E.M. of three experiments performed in triplicate. $^{**}P < 0.005$ two-tailed, unpaired *t*-test.

clophilin D interact with these two cyclosporin A derivatives with the same affinity constants.

Fig. 4 shows that both MeVal-4-cyclosporin and D-MeVal-11-cyclosporin afforded some protection at 6 h but their effects were no longer significant after 24 h of glutamate exposure (either PI/calcein or LDH release assays). We have also tested the effects of these analogues on adenine nucleotide levels. Again, cyclosporin A and both of its derivatives, MeVal-4-cyclosporin and D-MeVal-11-cyclosporin, decreased the loss of ATP + ADP at 2 h after glutamate addition (ATP + ADP levels in glutamate-challenged cultures were 80.3 ± 4.3%, 95.2 ± 6.5%, 91.7 ± 5.9% or 93.4 ± 5.4% of those in cultures treated with vehicle alone or in the presence of 5 μM of the corresponding cyclosporin derivative, cyclosporin A, MeVal-4-cyclosporin and D-MeVal-11-cyclosporin, respectively, (P = 0.07, 0.1 and 0.07, respectively, two-tailed t-test, n = 8–10). As for cyclosporin A, neither of these derivatives modified, by itself, adenine nucleotide levels (results not shown). Since D-MeVal-11-cyclosporin and MeVal-4-cyclosporin are less effective than cyclosporin A against glutamate excitotoxicity, and, even though the first does not bind cyclophilin, both have similar protective effects, these results suggest that the effects of cyclosporin A cannot only be attributed to its interaction with cyclophilin and inhibition of permeability transition pore opening, but possibly take place also via calcineurin and/or additional targets.

3.4. The role of nitric oxide (NO) and poly (ADP-ribose) polymerase in glutamate-induced neuronal death

The loss in ATP levels during glutamate-induced excitotoxicity could also result from NAD depletion by poly (ADP-ribose) polymerase. Zhang et al. (1994) have proposed that NO production by NO synthase (NOS) activates poly (ADP-ribose) polymerase resulting in NAD and ATP depletion. Since phosphorylation of NOS inhibits its catalytic activity, calcineurin inhibitors might functionally inhibit NO formation, poly (ADP-ribose) polymerase activation and ATP depletion, by enhancing NOS phosphorylation (Snyder and Sabatini, 1995; Snyder et al., 1998).

To study whether calcineurin action on NOS and poly (ADP-ribose) polymerase activation was involved in cyclosporin A protection against glutamate-neurotoxicity, we have tested the effects of NOS and poly (ADP-ribose) polymerase inhibitors. Fig. 5A, B shows that both the NOS inhibitor N^G-nitro-L-arginine methyl ester, L-NAME (500 μM), and 3-aminobenzamide (5 mM), an inhibitor of poly (ADP-ribose) polymerase (Cookson et al., 1998) diminished glutamate-induced neuronal death at 6 and 24 h after glutamate exposure. The poly (ADP-ribose) polymerase inhibitor also reduced glutamate-induced fall in ATP and ATP + ADP occurring 2 h after glutamate addition (Fig. 5C and D). Therefore, the results show that NOS and poly (ADP-ribose) polymerase activity are required for glutamate-induced ATP drop and neuronal death.

4. Discussion

We have found that exposure of cerebrocortical neurons to glutamate induces a rapid fall (within 30 min) in ATP, ATP + ADP, and the ATP/(ATP + ADP) ratio that preceded neuronal necrosis. In these same neurons, glutamate elicited the production of O_2^-, which was evident at about 15 min after glutamate exposure. The site of glutamate-induced O_2^- production in cortical (Carriedo et al., 1998) or hippocampal neurons (Bindokas et al., 1996; Sengpiel et al., 1998) and cerebellar granule cells (Castilho et al., 1999) is thought to be the mitochondria.

The presence of Ca^{2+} and the production of reactive oxygen species in mitochondria are among the events that trigger permeability transition pore opening (Halestrap et al., 1997). Conversely, pore opening may also create a feed-forward mechanism to further increase reactive oxygen species production (Zamzami et al., 1997). In our cultures, the pore blocker cyclosporin A was unable to prevent glutamate-induced O_2^- production. These results indicate that pore opening upon glutamate challenge, if it occurs, must be either downstream or independent of O_2^- production. On the other hand, cyclosporin A was a potent inhibitor of ATP or ATP + ADP loss and necrotic glutamate-induced neuronal death. While these results open up the possibility that pore opening might be the origin of ATP loss, the finding that cyclosporin A derivatives that do not bind calcineurin afforded smaller protection against cell death than cyclosporin A suggest the participation of calcineurin and/or other cyclosporin A targets. Thus, it appears that cyclosporin A reduction of glutamate excitotoxicity of cerebrocortical neurons does not involve mitochondrial cyclophilin and the permeability transition pore as main targets, but also calcineurin. Interestingly, Castilho et al. (1998) and Nicholls and Budd (1998) have reported that cyclosporin A and the putative pore blockers MeVal-4-cyclosporin and bongkreic acid had very small effect on glutamate-induced Ca^{2+} deregulation in cerebellar granule neurons, a process associated with reactive oxygen species production and neuronal death, which involves mitochondria (Castilho et al., 1999). On the other hand, it is worth noting that cyclosporin A and its derivatives were all more effective in preventing acute than delayed neuronal death. Whether this is due to a slowdown or reduction of necrosis remains to be established.

The loss of ATP in the course of glutamate excitoxicity has been also attributed to NAD depletion by the nuclear enzyme poly (ADP-ribose) polymerase (Zhang et al., 1994), a process triggered by Ca^{2+}-dependent NO production. NMDA-induced neuronal death of primary hippocampal or cortical neurons is reduced by NOS and poly (ADP-ribose) polymerase inhibitors (Dawson et al., 1996; Skaper et al.,

1998). Importantly, the analysis of neuronal NOS (nNOS) null mice has shown that NMDA-induced neurotoxicity is markedly attenuated in cortical neurons derived from these animals (Dawson et al., 1996). In addition, Heller et al. (1995) have provided evidence that poly (ADP-ribose) polymerase activity is responsible for NAD depletion in islet cells in response to DNA damage induced by NO, since NAD loss was substantially blocked in poly (ADP-ribose) polymerase null mice. This work also showed that protection by 3-aminobenzamide against NAD loss was due to its inhibitory effect on poly (ADP-ribose) polymerase and not to other pharmacological properties of 3-aminobenzamide, since it was no longer protective in poly (ADP-ribose) polymerase deficient cells (Heller et al., 1995).

In the cerebrocortical neuronal cultures used in this work, the poly (ADP-ribose) polymerase inhibitor 3-aminobenzamide reduced the early drop in adenine nucleotides and delayed neuronal death induced by glutamate, an effect also exerted by the NOS inhibitor L-NAME. Phosphorylation of NOS inhibits its catalytic activity, and cyclosporin A and other calcineurin inhibitors can inhibit NO formation, poly (ADP-ribose) polymerase activation, and ATP depletion by enhancing NOS phosphorylation (Dawson et al., 1996; Snyder et al., 1998). Therefore, these results suggest that the cyclosporin A-sensitive pathway leading to ATP loss and cell death involves calcineurin in addition to mitochondrial cyclophilin. As found for cyclosporin A, NOS and poly (ADP-ribose) polymerase inhibitors were more effective in preventing acute than delayed neuronal death. On the other hand, this does not imply that the calcineurin-NOS-poly (ADP-ribose) polymerase pathway is the only target of immunophilin ligands, such as cyclosporin A and FK506, since unlike NOS and poly (ADP-ribose) polymerase inhibitors, the calcineurin inhibitor FK506 was still protective against NMDA-excitotoxicity in nNOS-deficient mice (Dawson et al., 1996). A new calcineurin target that may be involved in apoptosis is BAD (Wang et al., 1999). In hippocampal neurons, glutamate triggers mitochondrial targeting of BAD, enhancing its heterodimerization with anti-apoptotic Bcl-2 proteins and promoting apoptosis. These processes are induced by Ca^{2+} and involve calcineurin-dependent dephosphorylation of BAD (Wang et al., 1999).

In conclusion, permeability transition pore opening may be involved in neuronal death induced by ischaemia/reperfusion (Kristián and Siesjö, 1996) and in a related paradigm, hypoglycemic insult, as this is protected by cyclosporin A derivatives that act exclusively on mitochondrial cyclophilin and not on calcineurin (Friberg et al., 1998; Khaspekov et al., 1999). However, our results indicate that protection by cyclosporin A of glutamate excitotoxicity in cortical neuronal cultures is related to inhibition of calcineurin and possibly other cyclosporin A targets in addition to direct effects on mitochondrial cyclophilin and the permeability transition pore. A better understanding of these targets would be of great help to develop adequate strategies to rescue injured neurons.

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