Calcineurin and mitochondrial function in glutamate-induced neuronal cell death

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Abstract We have previously reported that glutamate can trigger a succession of necrosis and apoptosis in cerebellar granule cells (CGC). Since specific blockers of the *N*-methyl-D-aspartate (NMDA) receptor channel prevented both types of cell death, the role of Ca^{2+}-dependent processes in the initiation of glutamate toxicity was further investigated. We examined the possible involvement of mitochondria and the role of the Ca^{2+}/calmodulin-regulated protein phosphatase, calcineurin, in the development of either type of cell death. Cyclosporin A and the more selective calcineurin inhibitor, FK-506, prevented the development of both early necrosis and delayed apoptosis. In addition, cyclosporin A prevented the collapse of mitochondrial membrane potential observed during the exposure to glutamate and the concomitant necrotic phase. When CsA was added immediately after glutamate removal, it also prevented delayed apoptosis of neurons that had survived the necrotic phase. Altogether, these results suggest the involvement of calcineurin and a role for mitochondrial deenergization as early signals in neuronal apoptosis induced by glutamate.

Key words: Cell death; Neuron; Glutamate; Mitochondria; Calcineurin

1. Introduction

Evidence from both in vitro and in vivo studies has suggested a role for the excitatory amino acid, glutamate, in several neurologic disorders ranging from acute insults, such as stroke, hypoglycemia and epilepsy, to chronic degenerative states, such as Huntington's disease and the AIDS dementia complex [1]. Overstimulation of glutamate receptors leads to sustained Ca^{2+} influx, predominantly through the NMDA receptor subtype. The resulting intracellular Ca^{2+} overload activates Ca^{2+}-mediated downstream mechanisms and eventually neuronal death [2]. The type of cell death in glutamate-treated neurons has been studied both in primary cultures in vitro [3–5] and in ischemia models in vivo [6–9], and it has been demonstrated that both necrosis and apoptosis take place. Necrosis leads to rapid destruction of neurons associated with cellular and organelle swelling, energy depletion and membrane destruction. Apoptosis, on the other hand, is an active cellular process characterized by chromatin condensation, DNA fragmentation and the formation of 'apoptotic bodies' [10–12]. The degree of apoptosis occurring in vivo is frequently underestimated, since secondary necrosis and phagocytosis of apoptotic cells take place [13]. However, an apoptotic component of cell death in the ischemic brain has been suggested by experiments showing DNA fragmentation in the ischemic tissue [6–9]. We have recently shown that both necrosis and apoptosis occur in cerebellar granule cells (CGCs) treated with glutamate [5]. Many neurons died rapidly by necrosis during and shortly after the glutamate insult. The surviving population later underwent chromatin condensation and DNA fragmentation, which are typical of apoptosis. Both types of cell death were abolished in the presence of the NMDA receptor antagonist, MK-801. In the same study, we demonstrated that mitochondria play an important role in glutamate-induced neuronal death [5]. During glutamate exposure (30 min), we detected a rapid loss of the mitochondrial membrane potential in the entire cell population. In more sensitive neurons, massive collapse of membrane potential was associated with energy depletion and necrosis, in other neurons that, at least in part, retained membrane potential, apoptosis progressed.

Mitochondria can actively transport Ca^{2+} into the matrix via a uniporter driven by the electrochemical proton gradient [14]. Ca^{2+} accumulation in the mitochondrial matrix leads to inner membrane permeability transition. The permeability transition is characterized by mitochondrial swelling, loss of membrane potential and unspecific release of ions and small molecules, including proteins, through a proteinaceous pore [15,16]. Therefore, we decided to investigate whether the observed disturbance of the mitochondrial membrane potential during excitotoxicity was associated with mitochondrial permeability transition. For these studies we used cyclosporin A (CsA), which prevents the opening of the proteinaceous pore [17–19]. However, cyclosporin A, in complex with the immunophilin, cyclophilin, is also a potent inhibitor of the Ca^{2+}/calmodulin-regulated protein phosphatase, calcineurin [20]. Calcineurin has a high affinity for Ca^{2+} and a broad substrate specificity [21]. Thus, we also investigated the effects of FK-506, a more selective calcineurin inhibitor, which protects against necrosis in cortical cultures [22] and in cultures of CGCs [23] by mechanisms different from those sensitive to CsA.

2. Materials and methods

2.1. Chemicals

Cyclosporin A was from Biomol Feinchemikalien (Hamburg). FK-506 was obtained from Fujisawa Pharmaceuticals. Okadaic acid, N^G-nitro-L-arginine, propidium iodide and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (St. Louis, MO), whereas 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) was from Molecular Probes (Eugene, OR). All other chemicals were of the highest available grade of purity and obtained from local suppliers.

2.2. Cell cultures and treatments

CGCs were isolated from 7 day old rats and cultured as previously described [24]. Briefly, CGC (0.25×10^6 cells/cm^2) were cultured in BME medium (Gibco) supplemented with 10% fetal calf serum,

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25 mM KCl and 0.5% (v/v) penicillin/streptomycin. To prevent the growth of glial cells, cytosine arabinoside (10 M) was added 48 h after seeding. CGC were used after 8–9 days in culture and were exposed to glutamate in a Locke solution (134 mM NaCl, 25 mM KCl, 4 mM $NaHCO_3$, 5 mM HEPES, 2.3 mM $CaCl_2$, 5 mM glucose) for 30 min. Thereafter, cells were reincubated in the culture medium. Inhibitors were present 15 min before, during, and 1 h after exposure, added at the end of the exposure to glutamate, or added 1 h after the exposure to glutamate (see figure legends for details).

2.3. Cell viability

Our previous findings [5] have shown that loss of MTT metabolizing activity [25] correlates with rapid neuronal lysis during exposure of CGCs to glutamate. Thus the MTT assay could be used here as an indicator of neuronal cell death by necrosis.

2.4. Detection of apoptotic cells

Cells were fixed in methanol and stained with propidium iodide as described in [5]. The cells were imaged and scored under a BioRad MRC 600 confocal microscope, using the 488 nm excitation wavelength of a krypton/argon laser.

2.5. Measurements of mitochondrial membrane potential

In living cells 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) exists either as a green fluorescent monomer (emission: 527 nm) at depolarized mitochondrial membrane potentials or as an orange-red fluorescent J-aggregate (emission: 590 nm) at hyperpolarized membrane potentials (negative to −140 mV) [26]. JC-1 undergoes a reversible shift in emission as more J-aggregates form with increasingly negative mitochondrial membrane potential. Neurons cultured on glass coverslips were loaded with JC-1 (1.0 μg/ml) for 20 min at 37°C in culture medium containing 2% fetal bovine serum. Cells were perfused with a Locke solution and exposed to glutamate or the other treatment under a BioRad MRC 600 confocal microscope. Confocal dual emission images were collected as described in detail elsewhere [5].

3. Results

As shown in Fig. 1, CsA (100 nM) protected CGCs from both necrosis and apoptosis. CsA prevented neuronal necrosis when added before, but not after the exposure to glutamate. This is in agreement with our previous observation that necrosis develops during and immediately after the exposure to glutamate [5]. In contrast, CsA protected from apoptosis also when the addition was performed immediately after the exposure to glutamate (Fig. 1). CsA was ineffective, if added 1 h after glutamate removal. The presence of CsA during glutamate exposure protected cells from loss of mitochondrial membrane potential (Fig. 2) as detected by confocal microscopy using the fluorescent indicator JC-1.

CsA is also an inhibitor of calcineurin activity, hence its effects on glutamate-induced neuronal death may not have been solely related to a decreased transition pore permeability. To determine the possible involvement of calcineurin in glutamate-induced necrosis and apoptosis we used a more selective calcineurin inhibitor, FK-506. FK-506 (100 nM) protected CGCs from both necrosis and apoptosis (Fig. 3), whereas okadaic acid, a generic inhibitor of protein phosphatases 1 and 2A (but not of calcineurin), had no effect on cell

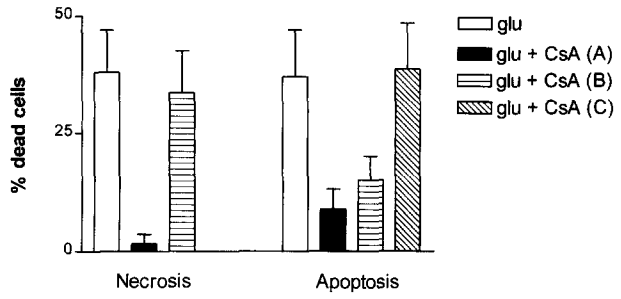

Fig. 1. Cerebellar granule cells treated with 100 μM glutamate (glu) in the presence or absence of 100 nM cyclosporin A (CsA). Necrosis was estimated by the impairment of MTT metabolism and expressed as a percentage. In this system, loss of MTT metabolism correlates with the rapid loss of membrane integrity, swelling and release of intracellular debris typical of necrosis [5]. Results are the mean ± S.D. ($n = 3$–5). Apoptosis was estimated by counting neurons with condensed apoptotic nuclei. Results are the mean ± S.D. ($n = 3$). Inhibitors were present before, during and 1 h after exposure to glutamate (A), added directly after glutamate exposure (B) or added 1 h after glutamate exposure (C).

death (results not shown). Neuronal nitric oxide synthase (NOS), thought to mediate, at least in part, glutamate toxicity, is a substrate for calcineurin [22]. Calcineurin-mediated dephosphorylation of NOS leads to increased formation of nitric oxide (NO). However, in this system, endogenous NO generation was not apparently involved in cell death, since NOS inhibitors such as N^G-nitro-L-arginine (10 μM or 100 μM) did not protect CGC from the decrease in MTT metabolism associated with neuronal death (Table 1).

4. Discussion

Excessive stimulation of glutamate receptors leads to intracellular Ca^{2+} overload and cell death [2]. We recently demonstrated that the type of cell death in CGC cultures exposed to glutamate depends on mitochondrial function [5]. Here, we report that the presence of cyclosporin A or FK-506 during exposure to glutamate protected CGCs from both necrosis and apoptosis, suggesting a role for calcineurin in this system, in addition to mitochondrial signals. Calcineurin is a Ca^{2+}/calmodulin-regulated protein phosphatase with broad substrate specificity. Calcineurin substrates that could affect neuronal survival include NOS [22], transcription factors [27,28] and the NMDA receptor itself [29–33]. In this system, the lack of a protective effect of N^G-nitro-L-arginine rules out the involvement of endogenous NO production. Furthermore, neither CsA nor FK-506 had any apparent effect on the Ca^{2+} overload induced by glutamate (data not shown). This suggests that calcineurin substrates other than NOS or the NMDA receptor channel are involved here.

CsA is a high-affinity inhibitor of a pore in the matrix-delimiting mitochondrial membrane [15,34]. Pore opening or

Table 1
Cerebellar granule cells treated with 100 μM glutamate (glu) in the presence or absence of N^G-nitro-L-arginine (NA) or treated only with NA

	100 μM glu	glu+10 μM NA	glu+100 μM NA	10 μM NA	100 μM NA
MTT metabolism (% of control)	76 ± 2.9	70 ± 12	74 ± 2.5	126 ± 15	110 ± 12

NA was present 15 min before, during and up to 24 h after exposure. Cell viability was estimated as described in Figs. 1 and 3. Results are mean values ± S.D. ($n = 3$).

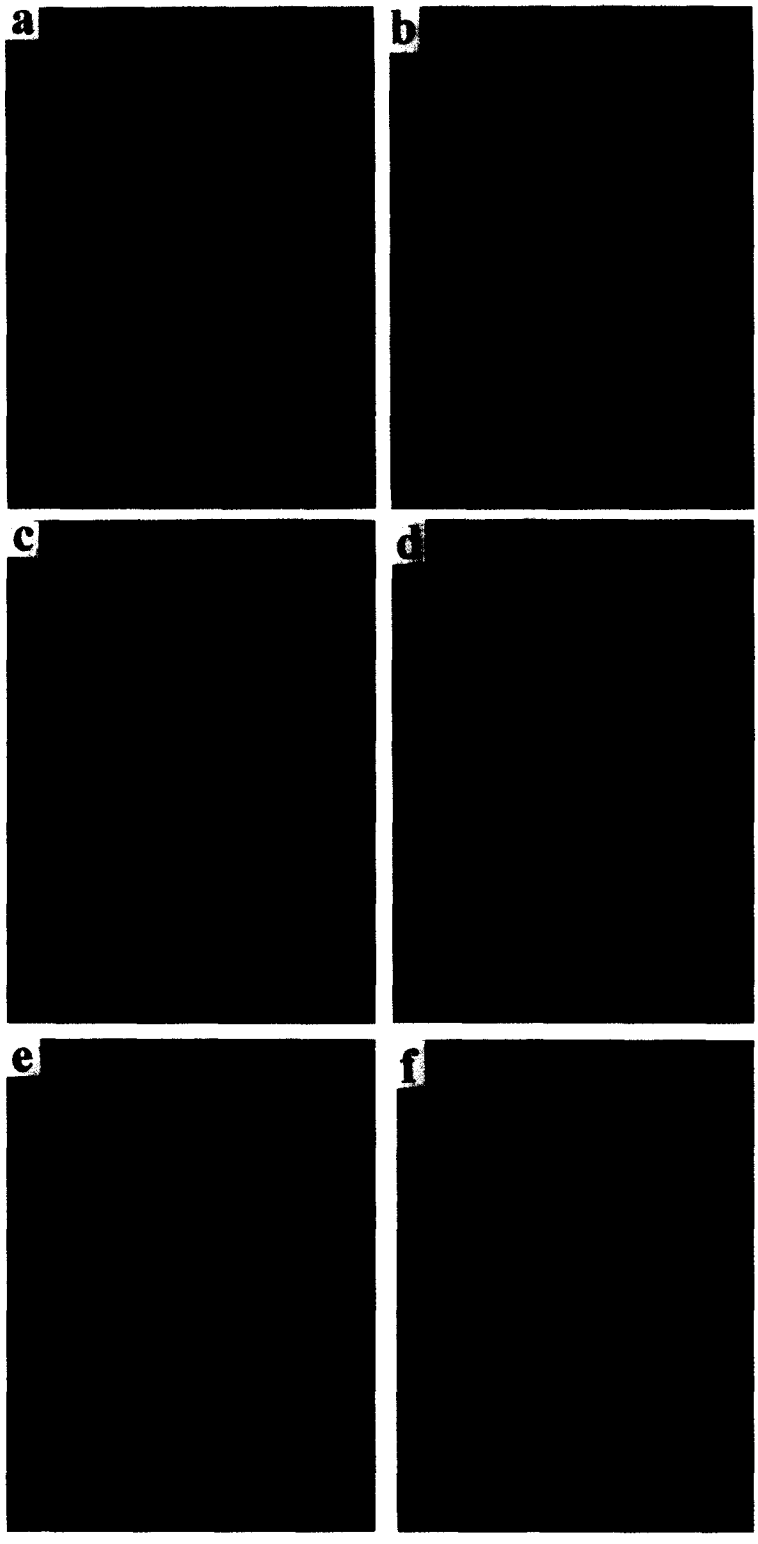

Fig. 2. Mitochondrial membrane potential in cells loaded with the fluorescent probe JC-1 (see Section 2) and exposed to 1 mM glutamate (glu) in the presence or absence of 500 nM cyclosporin A (CsA). Panel a: control 0 min. Panel b: control 30 min. Panel c: glu 0 min. Panel d: glu 30 min. Panel e: glu+CsA 0 min. Panel f: glu+CsA after 30 min.

permanence of the pore in an open state is favored by Ca^{2+} or depolarization. Thus, Ca^{2+} overload would allow permeability transition with the loss of matrix components. We found that in addition to inhibiting calcineurin, CsA protected from the loss of mitochondrial membrane potential during exposure to glutamate. Thus, CsA could either favor the conservation of the membrane potential and energy production per se or prevent the release of a mitochondrial-associated protein with a regulatory role in cell death. The first mechanism would be relevant for both necrosis and apoptosis. Nevertheless, cells surviving the initial necrosis caused by glutamate, which then undergo apoptosis, recovered mitochondrial membrane poten-

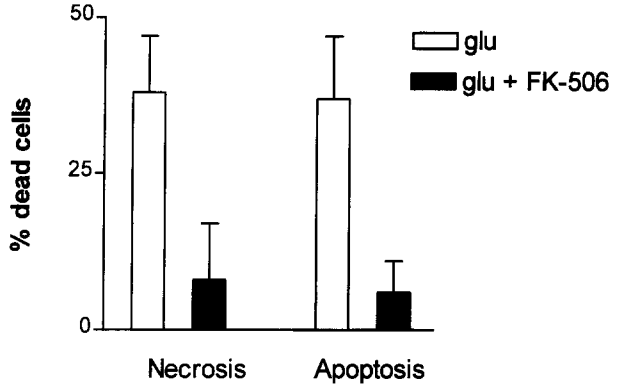

Fig. 3. CGCs treated with 100 μM glutamate (glu) in the presence or absence of 100 nM FK-506. Necrosis and apoptosis were estimated as described in the legend to Fig. 1. Results are the mean ± S.D. ($n = 3$–5) for necrosis and ($n = 3$) for apoptosis. FK-506 was added before the exposure to glutamate.

tial and energy charge also in the absence of CsA [5]. The second mechanism which is suggested by previous observations [35,36], would be relevant for the development of apoptosis. The findings that CsA prevented apoptosis when added immediately after exposure to glutamate, but not 1 h later, suggests that an irreversible event necessary for the progression of apoptosis occurs during this time. This may involve the release of a mitochondrial 'death factor' [36] from partially deenergized mitochondria. CsA positive effect on the mitochondrial membrane potential is in line with this assumption, although CsA and possibly FK-506 may in fact prevent upstream events involved in mitochondrial deenergization. At present we cannot distinguish between these two possibilities.

In conclusion, our results suggest that calcineurin activity and possibly the mitochondrial permeability transition are involved in the early phase of neuronal apoptosis elicited by glutamate. The possibility that calcineurin and a mitochondrial 'death factor' may interact on the pathway to neuronal demise by excitotoxins is under current investigation.

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