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Inhibition of the functional expression of *N*-methyl-D-aspartate receptors in a stably transformed cell line by cyclosporin A

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Abstract

The L(tk-) cell line L12-G10 stably transformed with the human *N*-methyl-D-aspartate (NMDA) receptor subunits NR1-1a/NR2A showed a Ca^{2+}-dependent increase in cell death, loss of mitochondrial membrane potential, and ATP depletion after agonist stimulation. Treatment of the cells with cyclosporine A (CsA) for 4 h reduced glutamate-induced cell death by 60% (IC_{50} of 7.1 μM). The immunophilin binding drug FK506 was not effective. Short preincubation with CsA for 10 min already decreased the glutamate-induced loss of mitochondrial membrane potential while the NMDA receptor function is not affected. However, pretreatment of the cells with CsA (30 μM) for 6 h reduced membrane associated NR1-1a protein amount by approximately 85%, whereas mRNA expression remained unaffected. These results suggest, that the cytoprotective effect of CsA in L12-G10 cells is due to the inhibition of the permeability transition pore on the one hand and to the inhibition of the expression of functional NMDA receptors by an additional posttranscriptional mechanism on the other hand.

Keywords: Cyclosporin A; Cyclophilins; Protein folding; NMDA receptor; L(tk-) cells; FK506

1. Introduction

Cyclosporin A (CsA) is a highly lipophilic, cyclic undecapeptide of fungal origin. Its immunosuppressive activity is mediated by the inhibition of calcineurin resulting in the block of transcriptional activity of early genes in T cell response such as IL-2 [1]. The intracellular protein targets of CsA are cyclophilins (CyPs) [2]. Beside CyPA which is an abundant, cytosolic protein found in all tissues and eukaryotic cells [3] other proteins like CyPB [4] containing an ER signal sequence or CyPM representing a mitochondrial matrix CyP [5] have been characterized. For the highly immunosuppressive drug FK506, a parallel signal transduction pathway has been described [6]. The target of FK506 is the FK506 binding protein (FKBP) [7].

The common step in the signal transduction of CsA and FK506 is the inhibition of the calcium/calmodulin-dependent serine phosphatase calcineurin by drug–immunophilin complexes [8]. In neurons, calcineurin has been shown to regulate the activity of native *N*-methyl-D-aspartate receptor (NR) channels [9]. Treatment with CsA or FK506 (200–500 nM) decreased the glycine insensitive desensitization after synaptic stimulation of NRs on rat hippocampal neurons. Thus, calcium entry mediated by native NRs in neurons seems to be limited by calcineurin activity. Besides, CsA has been described to prevent NR mediated neuronal cell death in vitro [10] and in vivo [11]. Overstimulation of NRs can lead to necrotic neuronal death as well as apoptotic events depending on the intensity of the overstimulation [12–15]. Intense stimulation of the NR with glutamate leads to increased cytoplasmatic calcium concentrations followed by mitochondrial calcium uptake and the opening of the mitochondrial permeability transition pore (PTP) spanning the inner and outer mitochondrial membrane [12] and results in necrosis of neuronal cells. Since the glutamate-induced neuronal death

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentration; CsA, cyclosporine A; CSS, controlled salt solution; CyP, cyclophillin; DM, dexamethasone; FKBP, FK506 binding protein; IL, interleukin; LDH, lactate dehydrogenase; NMDA, *N*-methyl-D-aspartate; NR, NMDA receptor; PPIase, peptidyl-prolyl isomerase; PTP, permeability transition pore

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requires mitochondrial calcium uptake [16,17] and is accompanied by a reduction of intracellular ATP pools [10] the opening of the PTP seems to play a key role in NR mediated excitotoxicity. CsA has been described to inhibit the PTP [18,19] and to delay mitochondrial depolarization induced by NMDA in cortical neurons (at 0.5–1 μM) [20]. It has been suggested that binding of the mitochondrial CyPM mediates CsA inhibition of the pore and is therefore involved in PTP function [21–23].

In the present study, we examined the effect of the two immunophilins cyclosporin A and FK506 on glutamate-induced NMDA receptor mediated cell death using a model of heterologously expressed NRs [24]. Different processes occurring after glutamate treatment were investigated in more detail. We report, that CsA but not FK506 prevents glutamate-induced cell death by inhibiting functional NMDA receptor expression.

2. Experimental

2.1. Cell line

Dexamethasone inducible expression of NR1-1a/NR2A in stably transformed L12-G10 cells has been described in detail [24]. In brief, expression of functional NRs was induced for the indicated time periods by 4 μM dexamethasone in the presence 100 μM ketamine to avoid cell death due to calcium overload. Removal of ketamine restores NR activity, and stimulation with L-glutamate/glycine in a controlled salt solution (CSS) containing 15 mM glucose, 120 mM normal saline, 50 mM potassium, 1.8 mM calcium, 4 μM dexamethasone, 25 mM Hepes adjusted to pH 7.6–7.8 results in a calcium-dependent necrosis.

2.2. Cytotoxicity assay

Cells were incubated with 4 μM DM for 20 h. Cytotoxicity assays were performed 1 h after stimulation with 100 μM L-glutamate/glycine. If CsA or FK506 (purchased from Alexis) were included, cultures were preloaded with different concentrations of immunophilin ligands for the indicated intervals. The determination of lactate dehydrogenase (LDH) release has been described in detail before [24]. In brief, cells were seeded in 96-well plates (15×10^3 cells per well), incubated with DM for 20 h and stimulated with 100 μM L-glu/gly in CSS. After incubation for 1 h at 37 °C, LDH release was measured in the supernatant by conversion of iodonitrotetrazolium chloride into a red formazan product whose absorption was measured at 490 nm in a Dynatech MR5000 ELISA-reader. Total LDH was determined after lysis of the cells with 0.1% Triton X-100. Cell death rates were expressed as a ratio of the absorptions of the supernatant and the lysate and are shown as mean ± S.D. ($n = 3$).

2.3. Determination of intracellular ATP levels

Dexamethasone (20 h) differentiated L12-G10 cells were stimulated in CSS containing 100 μM L-glutamate/glycine and 1.8 or 0.1 mM Ca^{2+}, respectively ($n = 3$). Differentiated L12-G10 cells cultured in CSS without agonists were used as control. Samples were taken prior to stimulation and after 10, 20, and 30 min. Cellular ATP content was determined as described in [25] using the CLS II ATP bioluminescence kit (Boehringer Mannheim). Samples were measured in a microplate luminometer LB 96V (EG&G BERTHOLD). The obtained relative light units were directly proportional to the ATP amount within the samples. ATP concentration is expressed as percentage of control samples and the values are given as mean ± S.D. ($n = 3$).

2.4. RT-PCR analysis

10^7 cells were differentiated for NR expression for 13 h. If included, CsA (30 μM) was added 4 h prior to harvesting. Total cellular RNA isolation, reverse transcription with $d(T)_{12–18}$ priming and subunit specific PCR were performed as described [24].

2.5. Analysis of NR cell surface expression by FACS

Dexamethasone treated L12-G10 cells were cultured in the presence or absence of CsA (30 μM) for up to 6 h. Cells were harvested by scraping and washed with ice cold PBS containing 1% FCS and 0.1% NaN_3. Cells were probed with N-terminal anti-NR1 antibody (1:50; Chemicon) followed by FITC-conjugated goat anti-mouse antibody (1:20; Sigma) at 4 °C. Cells were fixed with 1% formaldehyde. Analysis was performed with FACS Calibur (Becton Dickinson). For quantification the percentage of positive cells was used. Data are given as mean ± S.D. ($n = 3$).

2.6. Immunoblotting

Dexamethasone treated L12-G10 cells were cultured in the presence or absence of CsA (30 μM) for up to 6 h. Cultures were harvested by scraping and centrifugation for 10 min at $3000 \times g$. The resulting pellets were shock frozen in nitrogen and stored at −70 °C. For Western blot analysis, cell pellets were resuspended in 1 ml 10 mM Tris buffer pH 7.4 containing 1 mM PMSF and 1 mM EDTA, homogenized by sonification and chilled on ice. Membranes were obtained by centrifugation for 15 min at 4 °C and $13{,}000 \times g$. Pelleted membranes were resuspended by sonification in 50 μl of hypotonic buffer containing 10 mM Tris, 1 mM EDTA, 1 mM PMSF, and 0.2% Triton X-100. For preparation of whole cell lysates, cell pellets were directly homogenized in 50 μl of the hypotonic buffer using sonification. Aliquots corresponding to 0.5×10^6 of cells were subjected to electrophoresis, blotted to PVDF membrane and probed with N-terminal anti-NR1 antibody

(1:400; Chemicon). Primary antibody was detected with AP conjugated goat anti-mouse antibody (1:5000; Sigma). As control the housekeeping protein β-actin was determined with an anti-β-actin antibody (1:500; Santa Cruz Biotechnologies), followed by rabbit anti-goat antibody coupled to AP (1:5000; Sigma). Fluorescence detection was performed using ECF (Amersham) and fluorescent image analyzer FLA-3000 (Fuji Film). Band intensities were quantified by the Advanced Image Data Analyzer software (raytest Isotopenmeßgeräte GmbH) and data given as mean ± S.D. ($n = 3$).

2.7. Measurement of intracellular Ca^{2+} levels and mitochondrial membrane potential

Dexamethasone treated L12-G10 cells were cultured in the presence or absence of CsA (30 μM) for up to 6 h. Cells were harvested by scraping and loaded in complete medium at 37 °C with 4 μM Fura-2/AM for 45 min and 10 μM TMRE for 15 min, respectively. Cells were washed with PBS and resuspended in CSS containing 1.8 mM Ca^{2+} to a final concentration of 5×10^6 cells per milliliter. One milliliter cell suspension was transferred into a thermally controlled (37 °C) fluorometer (Aminco-Bowman Series 2) with continuous stirring.

For measurement of intracellular Ca^{2+} level, the fluorescence emission of Fura-2 loaded cells at 510 nm was measured after excitation at 340 and 380 nm, respectively. Intracellular Ca^{2+} levels were calculated according to the method of Grynkiewicz et al. [26]. Maximal fluorescence was obtained by lysing the cells with 1% Triton X-100, minimal fluorescence by chelating Ca^{2+} with 10 mM EDTA. The results are given as the change in intracellular calcium concentration.

For the determination of changes in mitochondrial membrane potential, the fluorescence emission of TMRE loaded cells at 574 nm was recorded after excitation at 549 nm. A loss of membrane potential results in an increased fluorescence signal [27].

3. Results

3.1. NR mediated Ca^{2+}-dependent cell death of L12-G10 cells

L12-G10 cells expressing the NMDA receptor subunits NR1-1a and NR2A show a calcium-dependent cell death after stimulation with 100 μM L-glutamate and glycine (Fig. 1). In the presence of physiological Ca^{2+} concentrations stimulation of the NR with L-glutamate/glycine leads to death of 73% of the cells, whereas control cells not stimulated with L-glutamate/glycine survived (data not shown). Drastic elevation of intracellular calcium levels leads to mitochondrial calcium overload, the induction of mitochondrial PTP, collapse of mitochondrial membrane potential, energy depletion, and subsequent cell death. Therefore, we determined changes in intracellular Ca^{2+}, in the mitochondrial membrane potential as well as changes of intracellular ATP levels during NR stimulation.

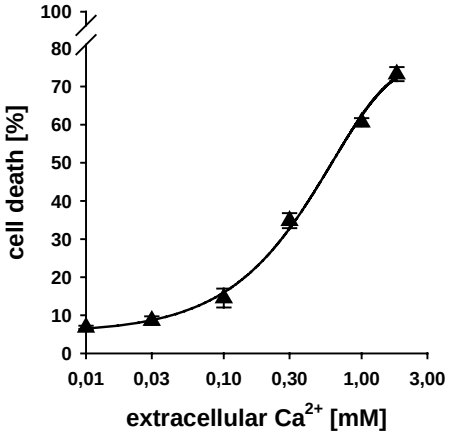

Fig. 1. Ca^{2+}-dependent cell death of L12-G10 cells after stimulation of NRs. L-Glutamate-induced excitotoxicity. NR expressing L12-G10 cells (20 h DM) were stimulated with 100 μM L-glutamate and glycine in CSS containing the indicated concentrations of Ca^{2+}. Cells cultured without L-glutamate and glycine, not showing significant cell death rates, were used as controls. Cell death rates were determined by LDH-assay 1 h after agonist stimulation with 100 μM L-glutamate and glycine.

Stimulation of NMDA receptors in L12-G10 cells leads to an influx of Ca^{2+} as demonstrated by calcium imaging experiments with Fura-2/AM. Fig. 2A shows that stimulation with 100 μM L-glutamate/glycine significantly increased $[Ca^{2+}]_i$ levels in dexamethasone-induced cells with a time course comparable to stimulation with the ionophor ionomycin. To show that this calcium influx induced the opening of the PTP resulting in a loss of mitochondrial membrane potential, cells were stained with tetramethylrhodamine ethyl ester. The accumulation of this fluorescent dye in mitochondria is driven by the mitochondrial membrane potential [27]. As shown in Fig. 2B, the Ca^{2+} influx after stimulation with 100 μM L-glutamate/glycine leads to a significant loss of mitochondrial membrane potential within 10 min. Furthermore, a strong ATP depletion was observed under physiological extracellular calcium concentrations (1.8 mM) whereas no significant changes were detected with low extracellular calcium concentrations (0.1 mM) (Fig. 2C).

3.2. Inhibition of NR-mediated cell death by CsA in a dose- and time-dependent manner

From these data we speculated that there is a NR-mediated damage of mitochondria caused by the calcium-induced opening of the mitochondrial PTP. Therefore, we investigated the PTP inhibitor CsA [18] for its cytoprotective effects in our excitotoxicity model. To exclude calcineurin-mediated effects, the calcineurin selective compound FK506 was included [28,29]. First, we excluded cytotoxic effects of the two compounds alone.

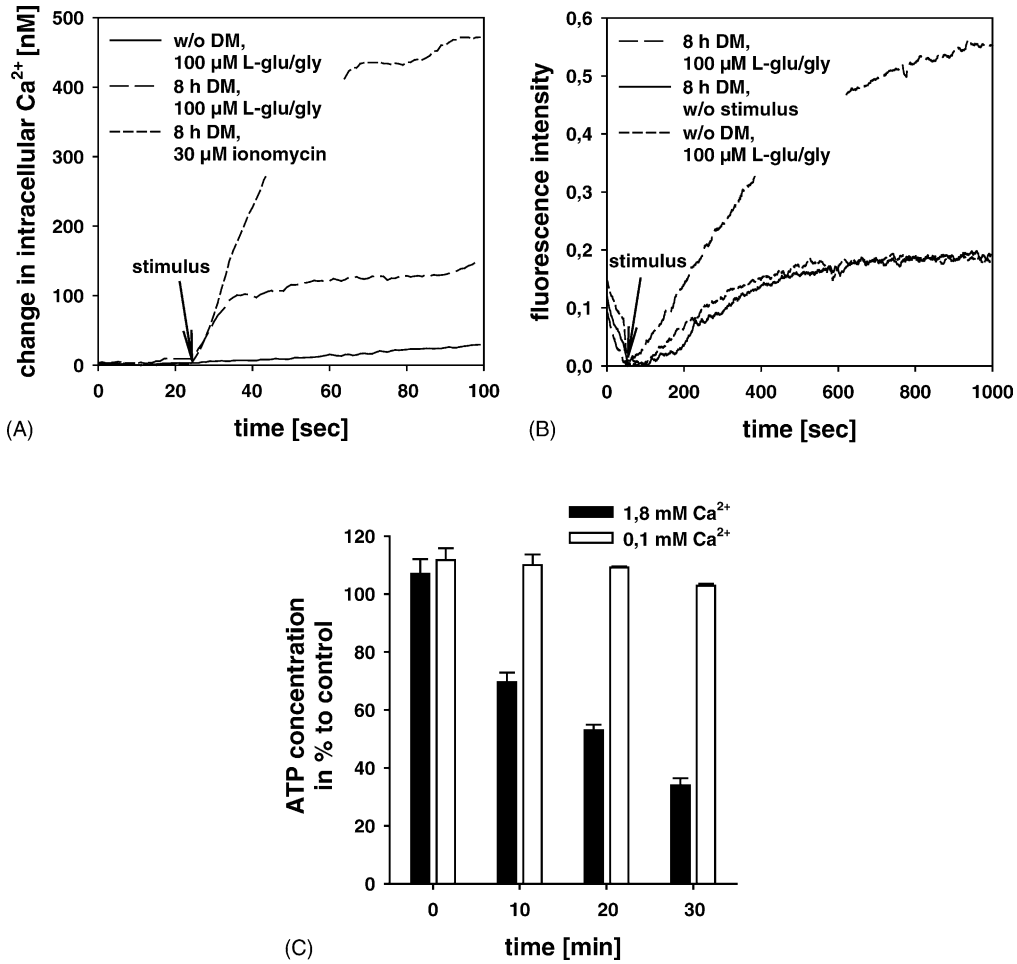

Fig. 2. NR stimulation in L12-G10 cells. L-Glutamate-induced signals in L12-G10 cells. (A) Change in intracellular Ca^{2+} of NR expressing L12-G10 cells (DM induction as indicated) was measured. Intracellular Ca^{2+} concentration at the time point of stimulation was approximately 200 nM. Cells were stimulated after 25 s with the indicated stimuli. (B) NR expression was induced with DM as indicated. Changes of the mitochondrial membrane potential were recorded after stimulation. (C) NR expression of L12-G10 cells was induced with DM for 20 h. Intracellular ATP depletion was measured at the indicated intervals after stimulation with 100 µM L-glutamate and glycine in the presence of 1.8 or 0.1 mM Ca^{2+}. Unstimulated cells were used as controls.

By trypan blue exclusion we could not observe significant cell death rates after incubation of the cells with up to 30 µM CsA or FK506 for up to 8 h (data not shown). Immunophilin ligands (30 µM) were preincubated prior to agonist stimulation for the indicated intervals. As shown in Fig. 3A, cytoprotective effects were obvious after a prolonged pretreatment of cells with CsA but not with FK506. FK506 did not exert cytotoxic effects alone as proven by stimulating cells preincubated with the drug in the presence of ketamine (data not shown). Maximum cytoprotection is obtained after 4 h and reduced the cell death rate by more than 60%. Furthermore, the cytoprotective effect of CsA shows a clear dose dependence with an estimated IC_{50}-value of 7.1 µM (Fig. 3B). Additional presence of CsA during the L-glutamate/glycine stimulation revealed similar potencies. The cytoprotective effect of CsA decreased if the preincubation interval was reduced. No significant prevention of cell death was observed with a short preincubation interval of 10 min.

The calcineurin selective compound FK506 displayed no significant changes in cell death rates. Additionally, the effective concentration range of CsA exceeds that for calcineurin inhibition by 10 to 100-fold. Therefore, we exclude an involvement of calcineurin in CsA-mediated cytoprotection.

Taken together, cytoprotective effects of CsA were only obvious after extended pretreatment with micromolar concentrations. Since CsA inheres a highly lipophilic structure, it is rather unlikely that a delayed uptake into the cells accounts for this observation. To further address the mechanism of CsA-mediated cytoprotection, the influence of CsA on different parameters such as NR expression, NR-mediated Ca^{2+} influx and loss of mitochondrial membrane potential were investigated.

3.3. Reduced expression of membrane associated key subunit NR1-1a by CsA through a posttranscriptional mechanism

To explore the time dependence of CsA mediated cytoprotection, a possible interference with the NR subunit expression was investigated. To analyze the influence of

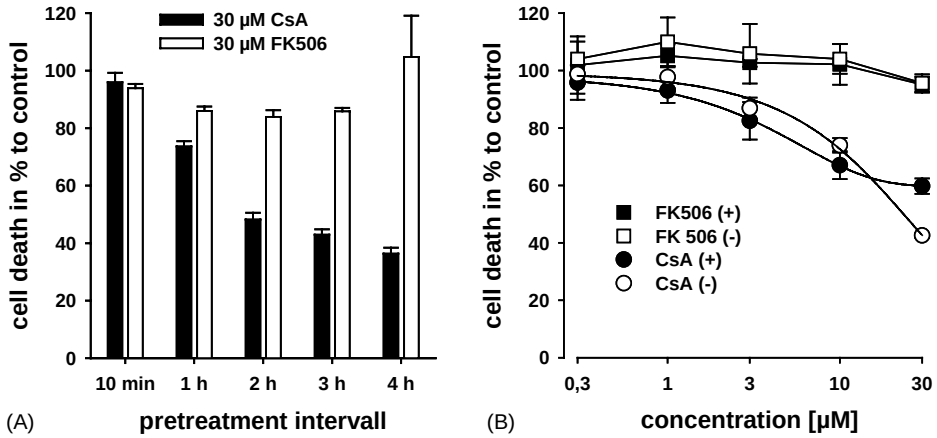

Fig. 3. Time dependence and dose response of CsA mediated cytoprotection. NR expressing L12-G10 cells (20 h DM) were loaded with the indicated concentrations of CsA or FK506 for various intervals (A: 10 min to 4 h; B: 4 h) and subsequently stimulated in CSS with 100 µM L-glutamate/glycine. Cell death rates were determined by LDH-assay 1 h after agonist stimulation. NR expressing L12-G10 cells without immunophilin ligands stimulated with 100 µM L-glu/gly were used as control. (A) Time dependence of CsA effects. Cultures were stimulated with L-glutamate/glycine after different times of pretreatment with indicated immunophilin ligand CsA or FK506. (B) Dose–response curves of immunophilin ligands. CsA or FK506 preincubated cultures (4 h) were stimulated with L-glutamate/glycine in CSS in the presence (+) or absence (−) of the respective preloaded immunophilin ligand.

CsA on mRNA expression of NR subunits, RT-PCR analysis was performed (Fig. 4A). L12-G10 cells were differentiated with 4 µM dexamethasone and 100 µM ketamine for 13 h. If included, 30 µM CsA was added 4 h prior to harvesting. Transfected but not induced L12-G10 cells were used as control. Treatment with dexamethasone for 13 h increased the steady state levels of both NR subunit mRNAs. The increased expression levels were not affected by prolonged treatment with 30 µM CsA. Hence, these results exclude an interference of CsA with NR mRNA expression.

On the other hand, pretreatment with CsA affected the amount of membrane associated NR protein of the key subunit NR1-1a. Treatment with 30 µM CsA for 4 h significantly reduced the expression of subunit NR1-1a protein as determined by FACS analysis (Fig. 4B).

We were further interested in the time course of the CsA effect on the surface expression of NR1-1a. As shown in Fig. 5, the surface expression of NR1-1a-subunit is slightly increased after short incubation with CsA but significantly decreased after a 4-h incubation period. Both, the surface expression revealed by FACS analysis (Fig. 5A) and the protein amount in the membrane fraction determined by immunoblotting (Fig. 5B) show the same time course. Similar results were found for NR1-1a level in whole cell lysates (data not shown). In accordance with the observation, that the maximal cytoprotective effect of CsA is reached after a 4-h incubation period, a 6-h incubation did not further diminish the protein expression (Fig. 5B).

Since the NR1 subunit represents the key subunit necessary for successful surface expression of the NMDA receptor complex [30], the described reduction of NR1-1a expression by CsA should alter the NR mediated calcium influx. Therefore, calcium imaging experiments with Fura-2/AM were performed. As shown in Fig. 5C, stimulation with 100 µM L-glutamate/glycine significantly increased $[Ca^{2+}]_i$ levels in dexamethasone-induced cells. This increase in inntracellular Ca^{2+} is only slightly

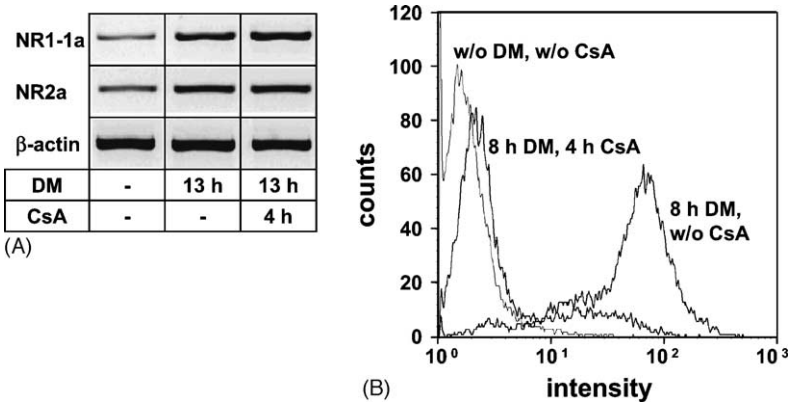

Fig. 4. Effects of CsA on NR mRNA expression and NR protein expression at the cell surface. Cells were incubated with 4 µM DM and 30 µM CsA for the indicated time periods. (A) RT-PCR analysis of NR1-1a and NR2A mRNA expression. mRNA expression of β-actin is shown as control. (B) FACS analysis of the NR1-1a surface expression detected with anti-NR1-1a antibody as described.

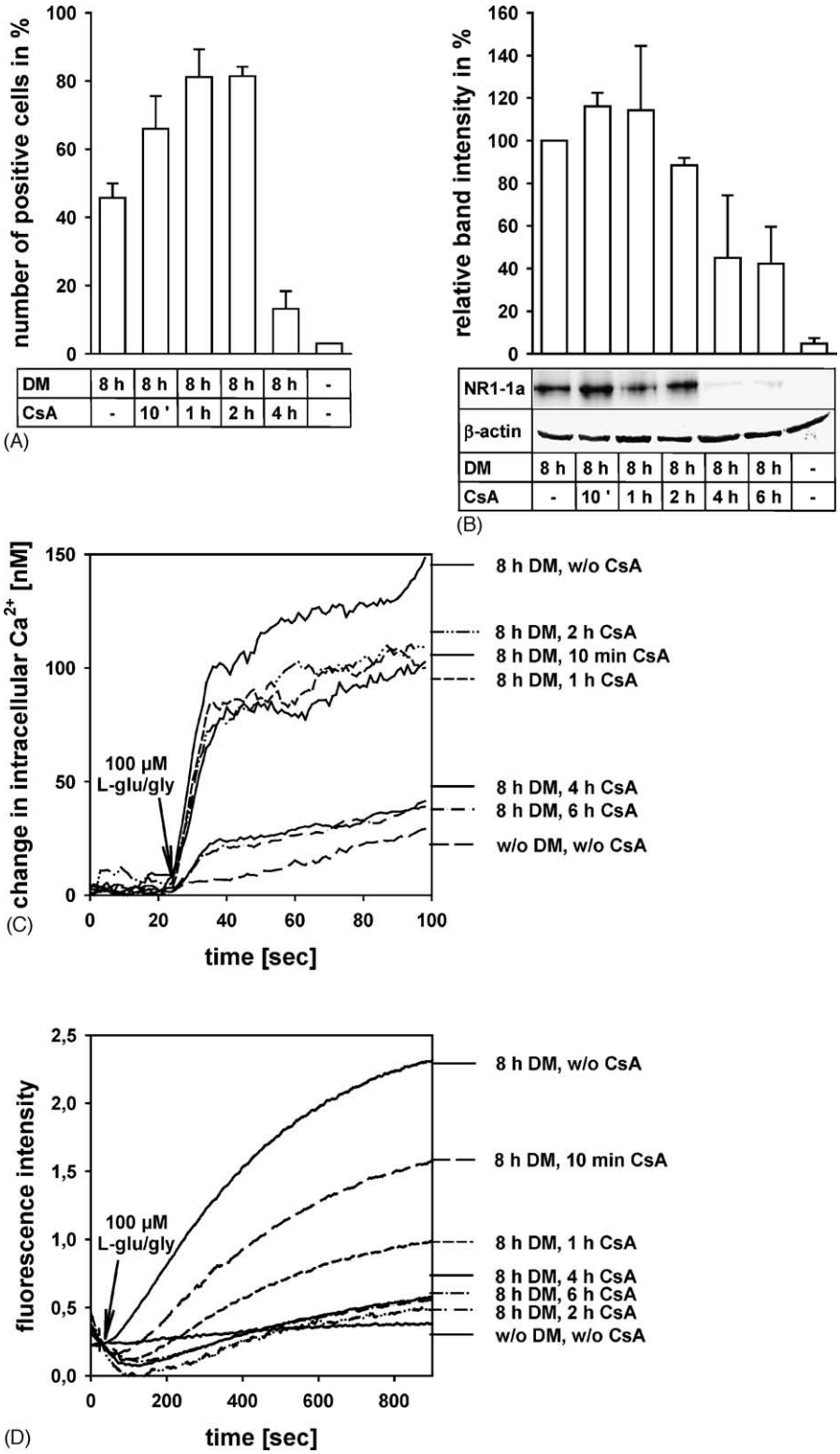

Fig. 5. Time course of CsA effects on NR expression, NR mediated Ca^{2+} influx and loss of mitochondrial membrane potential. Cells were incubated with 4 μM DM and 30 μM CsA for the indicated time periods. (A) FACS analysis of the NR1-1a surface expression. The number of cells, expressing NR1-1a at the cell surface, relative to the total cell number is shown. (B) Immunoblotting analysis of membrane expression of NR1-1a. The panel with NR bands represents one out of three experiments used for the quantification. β-Actin panel is shown as loading control. (C) Change in intracellular Ca^{2+} after stimulation of NR with 100 μM L-glu/gly. Intracellular Ca^{2+} concentration at the time point of stimulation (approximately 200 nM) was set to zero. (D) Loss of mitochondrial membrane potential after stimulation of NR with 100 μM L-glu/gly.

reduced by short CsA treatment (up to 2 h). In contrast, longer treatment with CsA almost completely abolished this influx. Again, a prolonged pretreatment is required to prevent the increase in intracellular calcium concentration.

Next, the loss of mitochondrial membrane potential due to opening of the PTP was investigated. As shown in Fig. 5D, treatment of the cells with CsA dramatically decreased this loss of membrane potential. Incubation periods of 10 min and 1 h already decreased this loss. However, for an almost complete block of loss of membrane potential a longer treatment with CsA of at least 2 h is necessary, while no further increase in efficiacy is observed upon longer CsA preincubation. The preservation of the mitochondrial membrane potential is thus faster occuring than the reduction of the NMDA receptot function.

4. Discussion

We could demonstrate, that overstimulation of NMDA receptors with glutamate in our cell system leads to influx of Ca^{2+}, lowering of the mitochondrial membrane potential as well as ATP depletion and finally to cell death. The time course of these events is comparable with data reported by others for cerebral cortex neuronal cultures [12,31].

Cyclosporin A was able to prevent cell death in neuronal cultures [29,32,33], but CsA concentrations needed in our cell system were significantly higher. In contrast, FK506, a more selective calcineurin inhibitor [28,29] also impeding cell death in neuronal cultures [32], was not effective to prevent cell death in our system, indicating that calcineurin inhibition does not play any role here. Pretreatment of the cells with CsA inhibited all investigated cellular glutamate responses with comparable time courses. Short preincubation with CsA for 10 min up to 2 h did not significantly influence the glutamate signalling whereas incubation with CsA for at least 4 h prevented influx of Ca^{2+} and finally cell death. However, short incubation periods with CsA of 10 min were sufficient to partially prevent the loss of mitochondrial membrane potential and consequently to reduce cell death. In neuronal cell cultures CsA treatment does protect the cells from NMDA receptor mediated cell death without interfering with intracellular calcium levels [28,32]. This mechanism is probably responsible for the relatively fast onset of cell protection. However, the more pronounced protection by CsA at longer preincubation times indicates that additional protective mechanisms exist that require prolonged exposure with the drug.

We could show that cell surface expression of NRs is decreased by treatment with cyclosporin A. A similar influence of CsA was found in rat thymus, where expression of metabotropic glutamate receptors was inhibited by CsA [34]. Although we observed a participitation of the PTP inhibition after short incubation with CsA as observed by others [32], our results clearly indicate, that prolonged treatment with CsA at micromolar concentrations decreases the amount of membrane associated NR protein by a posttranscriptional mechanism which seems to be responsible for the strong protective effect of CsA observed at longer time points. This finding might be explained by an interference with nascent protein folding. Beside its ability to inhibit calcineurin and PTP, CsA has been shown to inhibit the peptidyl-prolyl isomerase (PPIase) activity of its intracellular target cyclophilin [35]. PPIase-like enzymes function as molecular chaperones to facilitate protein folding, intracellular trafficking, and maintenance of multiprotein complex stability [36–38]. They accelerate nascent protein folding in-vivo by catalyzing the cis–trans isomerization of peptidyl-prolyl peptide bonds [39]. This isomerization is often the rate limiting step in the generation of native polypeptide conformation. There is evidence that CsA undergoes a cis–trans isomerization resulting in a trans-isomer which is slowly released from the active side of the enzyme [40,41]. In contrast to calcineurin inhibition, this competitive inhibition of PPIase activity requires inhibitor concentrations within the micromolar range, similar to the concentrations required here for inhibition of NR expression. This misfolding of the NR protein probably leads to retention of the protein within the endoplasmic reticulum followed by its rapid degradation [42–44].

Other ion channels have been proposed to be cyclophilin substrates. A prolonged treatment (16 h) with high CsA concentrations (IC_{50} of 11 μM) reduced the functional expression of Kir2.1 potassium channels in *Xenopus* oocytes [45]. Since the protein synthesis was not impaired by CsA, authors concluded that the functional expression of these channels is facilitated by the PPIase activity of cyclophilin. A similar hypothesis has been proposed for the functional surface expression of nicotinic acetylcholine receptor subunit α7 and a type 3 serotonin receptor in *Xenopus* oocytes which was reduced by prolonged CsA treatment (7 μM) for 4–7 days [46].

These findings are in good agreement with our results. In our excitotoxicity model cytoprotective effects of CsA were only obvious after prolonged treatment with concentrations in the micromolar range. The estimated IC_{50}-value of 7.1 μM is comparable to IC_{50}-values reported for inhibition of functional Kir2.1 potassium channel expression in *Xenopus* oocytes.

Hence, our data may suggest a crucial role of CyPs in the folding of nascent NR subunits. A participation of FKBPs in NR folding seems to be unlikely, since FK506 was not effective in prevention of cell death.

Acknowledgments

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