Cyclophilin-A is involved in excitotoxin-induced caspase activation in rat neuronal B50 cells

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Glutamate and the NO donor, nitroprusside, synergistically induced the death of B50 cells from a rat CNS-derived neuroblastoma cell line. With low [nitroprusside] (10 μM) both nitroprusside and glutamate were required. Under these conditions, nuclei became pyknotic and caspases were activated. The activities of caspase-3 and caspase-6 (effector caspases) were higher than those of caspase-8 and caspase-9 (initiator caspases). The activation of all four caspases was inhibited by cyclosporin A, with the order of susceptibility caspase-8 = caspase-9 = caspase-6 > caspase-3. To identify the possible locus of cyclosporin A action, we used an antisense oligodeoxynucleotide to suppress the level of cyclophilin-A to < 5 % of its control value. Cyclophilin-A suppression largely reproduced the inhibitory effects of cyclosporin A. These results provide the first indication that cyclophilin-A participates in the activation of the caspase cascade in neuronal cells, in particular in the form of cascade elicited by excitotoxic stimuli. It is concluded that neuroprotection by cyclosporin A against excitotoxin-induced apoptosis is, at least partly, due to inhibition of cyclophilin-A.

Key words: apoptosis, cyclosporin A, cyclophilin, nitric oxide, glutamate.

INTRODUCTION

Glutamate is a principal excitatory neurotransmitter in the mammalian brain. During periods of anoxia or ischaemia, however, glutamate accumulates massively and becomes neurotoxic. Glutamate excitotoxicity depends on excessive Ca^{2+} influx into the cell through the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor [1], and gives rise to a mixture of necrotic and apoptotic cells. The lethal mechanisms triggered by the Ca^{2+} influx remain unresolved. Proposed mediators of glutamate toxicity include Ca^{2+}-activated enzymes, such as the protease calpain [2] and nitric oxide synthase (NOS) [3]. NO is neurotoxic [4,5] possibly by inhibiting respiration, since it competes with O_2 at cytochrome oxidase and it can also nitrosylate and inactivate complex I of the respiratory chain [6]. Cellular Ca^{2+} overload gives rise to mitochondrial Ca^{2+} overload, and there are reports that mitochondrial Ca^{2+} accumulation is needed for glutamate toxicity [7]. This may reflect an excitotoxic requirement for superoxide [8], since mitochondrial production of superoxide is greatly increased by mitochondrial Ca^{2+} overload [9,10]. Mitochondrial Ca^{2+} overload and oxidative stress synergistically provoke opening of the mitochondrial permeability transition (PT) pore [11], which uncouples mitochondrial energy transduction and undermines maintenance of cellular ATP. PT pore opening may mediate Ca^{2+}-induced injury in the ischaemic heart and other tissues (reviewed in [12]) and has also been proposed as a neurotoxic mechanism ([13,14] and references therein).

With such an array of possible causes, one looks for selective means of testing for key events in excitotoxicity. Following the finding that cyclosporin A (CSA) protects against ischaemic injury in the heart [15,16], CSA was also found to suppress glutamate toxicity in some neuronal cell types, such as hippocampal neurons [13,17] and cortical neurons [18], but not in others, such as cerebellar granule cells [19]. CSA binds with high affinity to cyclophilins (CyPs), a family of proteins with distinct isoforms in various intracellular compartments. CSA protection probably means that one or other CyP has a key role in excitotoxicity. Indeed, the mitochondrial CyP-D isoform is a component of the PT pore. In addition, the Ca^{2+}-activated protein phosphatase, calcineurin, is potently inhibited by CyP–CSA complexes [20] so that CSA may also inhibit certain Ca^{2+}-mediated changes in protein phosphorylation. Rather more selectivity can be obtained using cyclosporins substituted at the 4-position of the ring, for example, N-methylvaline-4-cyclosporin (which is also protective [13]), since the substituent group prevents the CyP–cyclosporin complex from binding to calcineurin. The derivatives still bind, however, just as CSA does to a number of functionally diverse CyP isoforms (see the Discussion). The possible ramifications of CSA administration are, therefore, quite numerous, and it is often difficult to draw precise conclusions about the mechanism of CSA action.

In this study, we have adopted an alternative approach using an antisense oligodeoxynucleotide (ODN) targeted specifically against the CyP-A mRNA, the cytosolic isoform, in the rat B50 neuronal cell line. We show that the antisense ODN effectively depletes CyP-A from the cells and that CyP-A depletion mimics the action of CSA in inhibiting the activation of the apoptotic pathway by glutamate and NO. The results demonstrate, for the first time, that CyP-A plays a fundamental role in excitotoxin-induced apoptosis in neuronal cells.

EXPERIMENTAL

Maintenance and treatment of B50 cells

B50 cells from a rat neuroblastoma cell line (European Collection of Cell Cultures, Salisbury, Wilts., U.K.), were maintained under CO_2/air (1:19) at 37 °C in Dulbecco's Minimal Essential Medium with glutamax, supplemented with 10 % (w/v) fetal-calf serum and 50 μg/ml gentamycin. Cells were seeded either on to 22 mm

well diameter, multiwell plates (Techno Plastic Products, Switzerland) or, for microscopy, on to laminin-coated glass coverslips (22 mm diameter), at a density not exceeding 10^5 cells per well/coverslip, and were maintained under CO_2/air in the same culture medium. The medium was replaced on day 2. When appropriate, 1 μM phosphorothioate ODN was introduced on day 3. The ODN used was an 18-mer with the sequence, 5′-CATGGCTTCCACAATGCT. On day 4, the cells were treated with 100 μM glutamate, 10 μM glycine, 10–100 μM sodium nitroprusside (as indicated) in Locke's solution (134 mM NaCl, 25 mM KCl, 10 mM Hepes, pH 7.4, 2.3 mM $CaCl_2$, 5 mM glucose) for 30 min, after which the medium was replaced with culture medium (as above) and the cells were returned to the CO_2 incubator. Controls were treated with Locke's solution alone. Nuclear morphologies were visualized by staining with the fluorescent cell permeant Hoechst 33258 (Sigma), or the cell impermeant propidium iodide. Digital images were obtained with a CCD camera (Princeton Instruments, Trenton, NJ, U.S.A.) fitted to a fluorescence microscope (Olympus IX70, Olympus, Tokyo, Japan).

DNA fragmentation

Cells were extracted in 100 mM NaCl, 10 mM Tris/HCl, pH 8.0, 25 mM EDTA, 0.5 % SDS and incubated at 37 °C for 1 h. 5 μl of proteinase K (25 mg/ml) was added and the mixture incubated for a further 4 h at 50 °C. The mixture was extracted 4 times with phenol/chloroform/isoamyl alcohol (25 : 24 : 1, by vol.) and twice with chloroform. DNA was precipitated by adding 400 mM sodium acetate and two volumes of ethanol, followed by incubation on ice for 30 min, and centrifugation (12 000 g for 15 min at 4 °C). The DNA pellet was washed once with ethanol, dried, and resuspended in 10 mM Tris/EDTA, pH 8.0. The DNA was analysed on 1.8 % agarose gels.

Caspase assay

Cells were extracted in 10 mM Hepes, pH 7.2, 2 mM EDTA, 0.1 % (w/v) CHAPS, 5 mM dithiothreitol, 2 mM phenylmethylsulphonylfluoride, and 10 μg/ml each of pepstatin, leupeptin and aprotinin. Cell extracts were frozen and thawed 4 times and clarified by centrifugation (10 000 g for 30 min). Further freeze–thaw cycles did not change the caspase activities. Also, the same caspase activities were recovered when the medium contained 5 % (w/v) CHAPS as detergent and the freeze–thaw was omitted. Caspases were assayed in 10 mM Pipes, pH 7.4, 2 mM EDTA, 0.1 % CHAPS, 1 mM dithiothreitol, using the fluorescent 7-amino-4-trifluoromethylcoumarin (AFC) derivatives of the following caspase-selective substrates: Ac-DEVD-AFC (caspase-3), Ac-VEID-AFC (caspase-6), Ac-EITD-AFC (caspase-8) and Ac-LEHD-AFC (caspase-9). Protein was determined with the Bradford assay using BSA as the standard.

Assay of CyP-A

For immunodetection of CyP-A, cell extracts were subjected to sequential isoelectric focussing (IEF) and SDS/PAGE before blotting. Cell extracts were prepared and fractionated by IEF as described previously [21] alongside CyP-A as marker (purified from rat liver [22]). IEF strips containing CyP-A were then subjected to SDS/PAGE in the second dimension, again as described before [21]. The separated proteins were electroblotted and developed with polyclonal anti-(CyP-A) antibodies (Calbiochem, Nottingham, U.K.) and peroxidase-labelled goat anti-rabbit IgG. Bands were detected using the enhanced chemi-

luminescence kit (Amersham, Little Chalfont, Bucks, U.K.). For markers, p53 was detected with a monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and the mitochondrial adenine nucleotide translocase (ANT) with a polyclonal antibody raised against the peptide sequence YDEIKKYV in the N-terminal region of rat ANT (this laboratory).

For enzymic detection of CyP-A, cells were extracted with 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM Hepes, pH 7.2, containing the protease inhibitors pepstatin, chymostatin, leupeptin and aprotinin (all at 1 μg/ml). KCl (100 mM) was added to prevent electrostatic association of CyP-A with membranes, and extracts were centrifuged in a Beckman Airfuge (130 000 g for 10 min). Supernatants were assayed for the peptidylprolyl *cis-trans*-isomerase activity (PPIase) of CyP-A using the fluorescent substrate *N*-succinyl-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin. Reactions were conducted in 20 μl reaction volumes on the stage of a fluorescence microscope as described previously [21]. CyP-A activities refer to the PPIase activities in cytosolic fractions (see above) blocked by 1 μM CSA and this accounted for the bulk (< 90 %) of the cytosolic PPIase activity.

RESULTS

### Synergistic induction of B50 neuronal cell death by glutamate and NO

Many types of neuron die after continuous exposure to glutamate and the co-activator glycine (see the Introduction). The cells succumb by a variable proportion of necrosis and apoptosis, depending on cell type and the duration of exposure to glutamate [23,24]. In this study, however, B50 cells remained viable irrespective of the period of exposure to glutamate (up to 3 h) and the glutamate concentration (10 μM–1 mM). Neuronal cell death can also be brought about by NO ([5] and references therein); therefore we tested the neurotoxicity of the NO donor, sodium nitroprusside, both alone and in combination with glutamate plus glycine.

B50 cells were subjected to 100 μM glutamate, 10 μM glycine and 50 μM nitroprusside for 30 min, and examined 24 h later after nuclear staining with the cell permeant Hoechst 33258 (Figure 1). Approximately 35 % of the nuclei displayed a highly condensed morphology (Figure 1b, arrow), compared with < 1 % in untreated control cells (Figure 1a). The same cell culture was also stained with the plasma membrane-impermeant propidium iodide (results not shown). All the condensed nuclei were thus stained, together with about 21 % of the normal sized nuclei (evidently necrotic). There was negligible staining of control nuclei by propidium iodide (< 1 %). Thus the combination of glutamate plus NO was clearly neurotoxic.

Figure 2(A) shows the relationship between condensed nuclei and [nitroprusside] in a typical cell preparation. Nitroprusside was toxic, whereas glutamate alone was not. However, the neurotoxicity of NO was markedly enhanced by glutamate. With 12 μM nitroprusside, both nitroprusside and glutamate were required for nuclear condensation.

Caspase activation following exposure to glutamate and NO

Pyknotic nuclei (Figure 1b, arrow) are a feature of apoptotic cells. DNA fragmentation, another indicator of apoptosis, has been reported in some neuronal types during glutamate excitotoxicity [25,26]. However, as shown in Figure 2(B), we were unable to observe this in B50 cells despite clear DNA laddering in cells subjected to other means of inducing apoptosis, i.e. staurosporine and oxidative stress. The possible reason for the
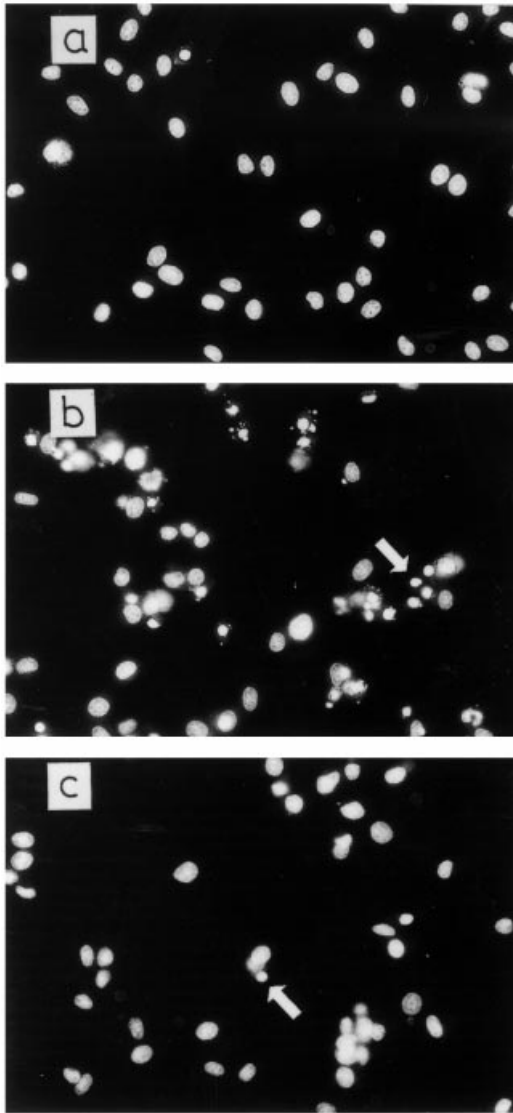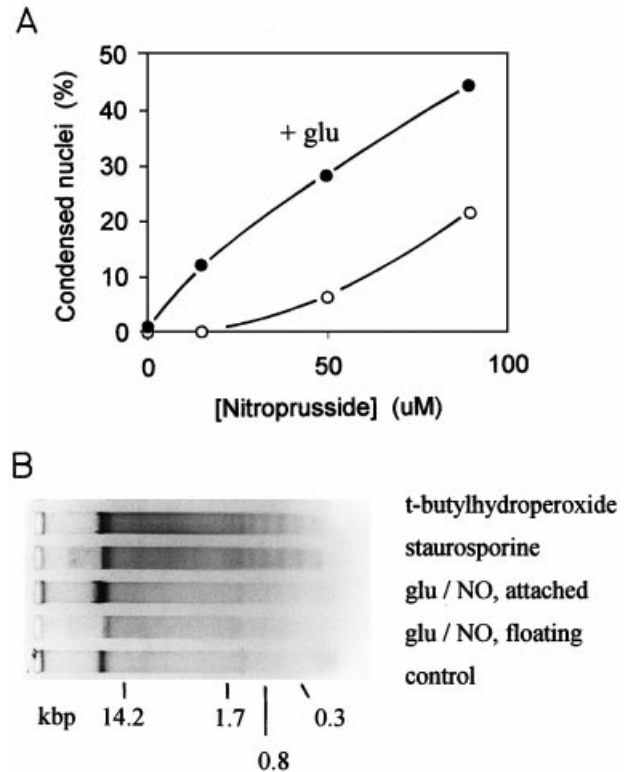
Figure 1   Nuclear morphologies after treatment with glutamate and nitroprusside in the presence or absence of CSA

B50 cell nuclei were stained with Hoechst 33258: (**a**) control cells; (**b**) cells stained 24 h after 30 min exposure to 100 μM glutamate and 50 μM nitroprusside; (**c**) as (**b**) except cells were treated (glutamate/nitroprusside) and incubated for 24 h in the presence of 1 μM CSA. Arrows indicate examples of pyknotic nuclei.

Figure 2   Nuclear condensation and DNA analyses

(**A**) B50 cells were treated with various [nitroprusside] for 30 min in the presence (●) or absence (○) of 100 μM glutamate (glu). Nuclei were stained 24 h later with propidium iodide, (**B**) cells were extracted 24 h after treatment with 100 μM glutamate/10 μM nitroprusside (glu/N0), 100 μM t-butylhydroperoxide, 1 μM staurosporine, or nothing (control). With glutamate/NO, some cells detached from the coverslips after 24 h, and DNA was extracted from both these floating cells and the attached cells. DNA was analysed alongside a Pst-1 digest of lambda DNA as marker, and some of the marker positions (kb) are given.

discrepant behaviour of glutamate-treated B50 cells may lie in the particular pattern of caspase activation, as described below.

As another criterion of apoptosis, we assayed various caspases. Caspases are expressed constitutively as inactive proenzymes and become activated after proteolytic cleavage. Caspase activation shows a hierarchy in that the 'effector' caspases, which bring about the morphological changes associated with apoptosis, are cleaved and activated by 'initiator' caspases. Here we examined two upstream caspases (caspase-8 and -9) and two downstream caspases (caspase-3 and -6). There was no significant caspase activation with 100 μM glutamate or 10 μM nitroprusside when these were added individually, but simultaneous exposure to 100 μM glutamate and 10 μM nitroprusside led to marked increases in caspase activity (Figure 3). Caspase-8 and -9 were

activated to a similar degree (approx. 6-fold after 24 h) and caspase-6 even more (approx. 20-fold after 24 h). By contrast, activation of caspase-3 was relatively small (approx. 3-fold after 24 h). Thus, whilst caspase-3 typically undergoes the major degree of activation in many cells under apoptotic stimuli (reviewed in [27]), it is rather poorly activated by glutamate/NO in comparison to other caspases. It should be noted, however, that the relatively low (fold) increase in caspase-3 activity in part reflects the higher basal activities of caspase-3, and that caspase-3 activities following glutamate/NO treatment were nevertheless higher than those of the other caspases assayed (see Figure 6).

Inhibition of glutamate plus nitroprusside-induced caspase activation by CSA

Figure 1(c) shows typical nuclear morphologies 24 h after exposure to glutamate plus NO in the presence of 1 μM CSA (CSA was retained throughout the 24 h period). The proportion of pyknotic nuclei was less than that observed in the absence of CSA (Figure 1b). In five such experiments (200–300 cells per experiment), the proportion of pyknotic nuclei after glutamate plus NO ($31\pm6\%$; means$\pm$S.E.M.) was decreased by 62% in the presence of CSA ($12\pm3\%$).
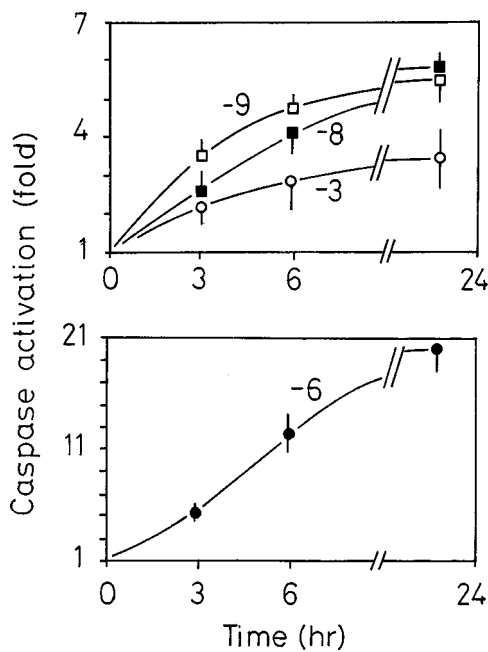
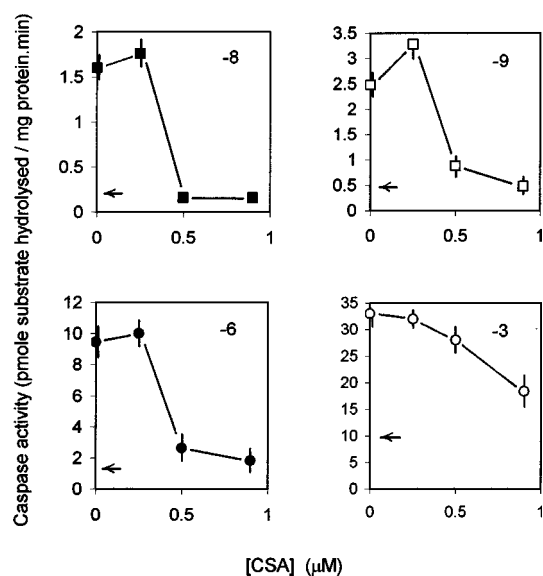**Figure 3   Caspase activation induced by glutamate/nitroprusside**

Cells were treated with 100 μM glutamate and 10 μM nitroprusside for 30 min and then extracted at the intervals shown. Extracts were assayed for caspase-8, -9, -6 and -3 as indicated. Values are means $\pm$ S.E.M. ($n = 4$).

Figure 4   Inhibition of glutamate/nitroprusside-induced caspase activation by CSA

Cells were preincubated with CSA at the concentrations shown for 15 min. The cells were then treated with 100 μM glutamate and 10 μM nitroprusside (see Experimental section). After 30 min, the medium was replaced with culture medium containing CSA (at the concentrations shown). Cell extracts were prepared 6 h later, and assayed for caspase-8, -9, -6 and -3 as indicated. Arrows indicate basal caspase activities without glutamate/nitroprusside treatment. Values are means $\pm$ S.E.M. ($n = 4$).

As shown in Figure 4, CSA also decreased the activities of caspase-8, -9 and -6 following glutamate plus NO insult, to basal levels (arrows). Caspase-3 activities were also progressively suppressed by increasing [CSA], but activation of this enzyme was clearly less susceptible to CSA than the other caspases assayed. Since caspase-8, -9 and -6 were greatly activated by glutamate and NO (Figure 3), and these activations were all substantially blocked by CSA (Figure 4), it appears that CSA is particularly effective against the profile of caspase activation elicited by glutamate/NO.

The involvement of CyP-A in CSA-inhibition of caspase activation

The principal cellular targets of CSA are the CyPs, a family of enzymes which catalyze the rotation of Xaa-Pro peptide bonds in protein substrates. The major isoform is CyP-A, resident in the cytosol. We examined whether CyP-A is the target of CSA, when it blocks caspase activation, by using an antisense ODN to suppress the expression of CyP-A in B50 cells. We have used the same antisense ODN previously to suppress CyP-A in rat heart cells [21], showing that the antisense ODN is complementary to an 18 base coding region of rat CyP-A mRNA, which is absent from the mRNAs for CyP-B and CyP-C (endoplasmic reticulum isoforms), CyP-D (mitochondrial isoform), and from CyP-40 and the CyP-domain protein Nup-358 (nucleus).

Initial experiments tested the efficiency of antisense-suppression of CyP-A in B50 cells. To ensure selective detection of CyP-A, cell extracts were dual-fractionated by IEF and SDS/PAGE before immunodetection. Figure 5(A) shows that after 24 h exposure to the antisense ODN, CyP-A was no longer immunodetectable in B50 cells. Other cellular markers, namely the amounts of p53 and the mitochondrial adenine nucleotide translocase (Figure 5A), and the activities of lactate dehydrogenase and malate dehydrogenase (not shown) were unaffected

by antisense treatment. In order to obtain a more quantitative assessment of the degree of CyP-A suppression, CyP-A was also assayed as CSA-sensitive PPIase activity in the cytosolic fraction of B50 cell extracts. In three experiments, antisense treatment removed approximately 95% of CyP-A activity (Figure 5B).

The effective suppression of CyP-A in B50 cells by antisense ODN offers an incisive means of investigating CyP-A involvement in caspase activation. As shown in Figure 6, antisense pretreatment largely prevented caspase activation brought about by glutamate plus NO, whereas the basal caspase activities were unaffected. The activation of caspase-8, -9 and -6 was inhibited to a similar degree (78–85%), and caspase-3 activation rather less (55%). In this series of experiments, cell extracts were
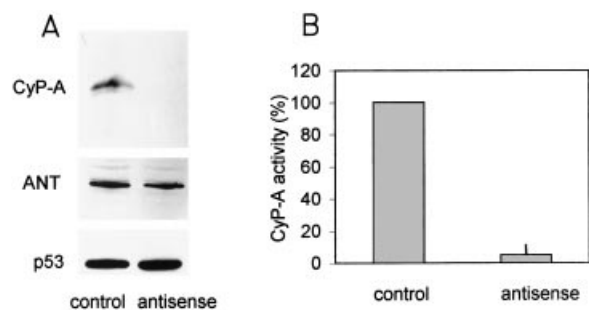
Figure 5   Suppression of CyP-A expression by the antisense ODN

Cells were incubated with or without 1 μM antisense ODN for 24 h. (**A**) Cells were extracted and assayed (immunoblots) for CyP-A, the mitochondrial adenine nucleotide translocase (ANT) and p53. (**B**) Cytosolic extracts were assayed for CyP-A peptidylprolyl *cis-trans*-isomerase activity. Antisense values are means $\pm$ S.E.M. ($n = 3$).
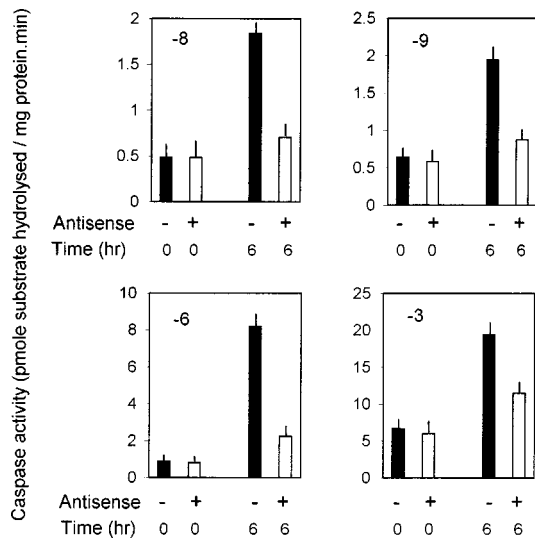
Figure 6 CyP-A suppression largely prevents glutamate/nitroprusside-induced caspase activation

Cells were either pretreated with the antisense ODN (+), or left untreated (−), exposed to 100 μM glutamate/10 μM nitroprusside for 30 min, and then extracted immediately (0 h) or 6 h later (6 h). The cell extracts were assayed for caspase-8, -9, -6 and -3 as indicated. Values are means ± S.E.M. ($n = 4$).

prepared 6 h after exposure to glutamate/NO, but similar degrees of antisense-inhibition were found in cell extracts prepared 3 h after glutamate/NO exposure, i.e. caspase-8, $86 \pm 4\%$; caspase-9, $77 \pm 5\%$; caspase-6, $82 \pm 6\%$; and caspase-3, $50 \pm 7\%$ (means ± S.E.M.).

It was important to establish that the decreased recoveries of caspase activity in antisense-treated cells reflected decreased caspase activation, rather than caspase loss from the cells as a result of necrosis. In fact, the proportion of necrotic cells (as

judged by propidium iodide staining) was relatively small at 6 h after glutamate plus NO, and was unchanged by antisense pretreatment, i.e. control cells, $6 \pm 3\%$ ($n = 4$) and antisense treated cells, $8 \pm 3\%$ ($n = 4$). All extracts assayed for caspase activities were also routinely assayed for the marker enzymes lactate dehydrogenase and malate dehydrogenase. Again, there was negligible ($< 6\%$) difference in the recoveries of lactate dehydrogenase and malate dehydrogenase in control and antisense-treated cells at 6 h after administration of glutamate and nitroprusside. We conclude that CyP-A suppression compromises caspase activation and that CyP-A probably has a major role in initiating the caspase cascade during glutamate plus NO excitotoxicity in B50 cells.

Caspase activation induced by oxidative stress and by staurosporine

The question arises whether CyP-A is involved uniquely in caspase activation associated with excitotoxicity, or whether it has a more general role. Accordingly, we investigated caspase activation brought about by other apoptotic stimuli, namely oxidative stress and staurosporine.

Preliminary experiments revealed that B50 cells quickly (3–5 h) became necrotic after addition of 0.5–1 mM t-butylhydroperoxide. With 100 μM peroxide, however, there was negligible necrosis ($< 10\%$) within 8 h, irrespective of antisense pretreatment. Under these conditions, all four caspases were markedly activated (Figure 7). Caspase-8, -9, and -6 were activated transiently, possibly reflecting consumption of the peroxide with time. These caspases were all activated approximately 14-fold after 4 h. Caspase-3 was activated even more, becoming approximately 10-fold more active than caspase-6. As with glutamate plus NO, peroxide-induced activation of caspase-8, -9, and -6 was substantially inhibited by suppression of CyP-A activity, whether brought about by antisense or by CSA. Again, caspase-3 activation was rather less sensitive to inhibition by antisense and CSA.

Initial experiments (results not shown) revealed that activation of all four caspases increased in an approximately linear manner
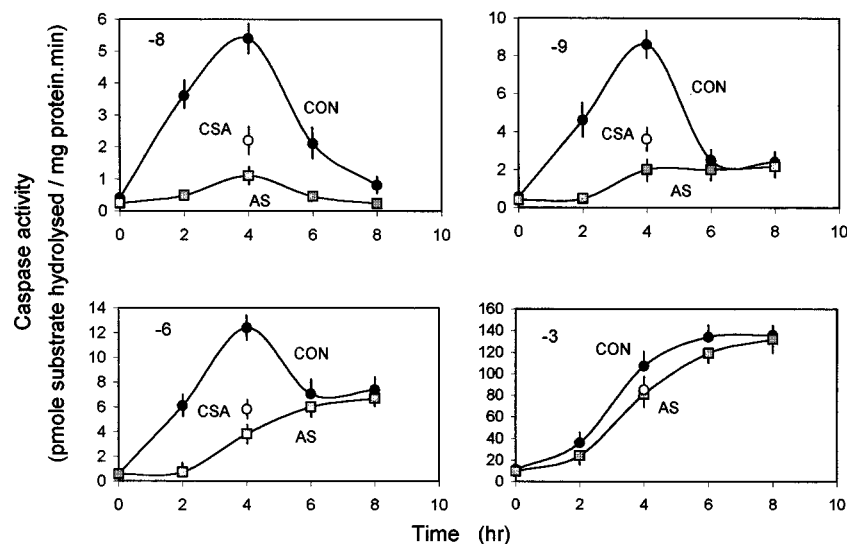
Figure 7 Peroxide-induced caspase activation, and its prevention by antisense pretreatment and by CSA

Cells were exposed to 100 μM t-butylhydroperoxide and extracted at the times indicated. Caspase-8, -9, -6 and -3 were assayed in control cells (CON) and antisense-treated cells (AS). CSA-treated (1 μM) cells were assayed at a single time point (4 h) as indicated (CSA). All values are means ± S.E.M. ($n = 4$).
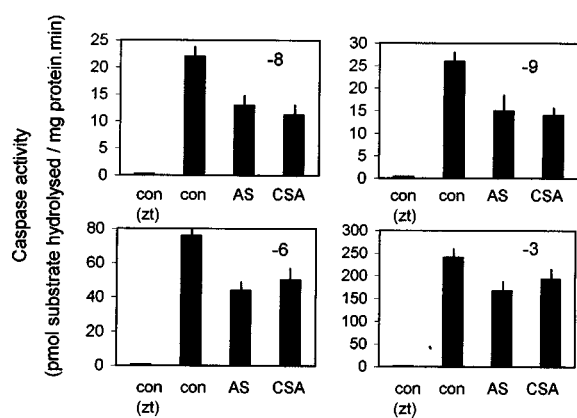
Figure 8   Staurosporine-induced caspase activation, and its partial prevention by antisense pretreatment and by CSA

Cells were exposed to 1 μM staurosporine and extracted 4 h later. Extracts were assayed for caspase-8, -9, -6 and -3 as indicated. Con (ZT), control cells at zero time exposure to staurosporine; Con, control cells at 4 h exposure to staurosporine; AS, antisense-treated cells at 4 h; and CSA, CSA-treated cells at 4 h. Values are means $\pm$ S.E.M. ($n = 3$).

with time, between 2 and 6 h after addition of the protein kinase inhibitor staurosporine. More extensive measurements were made, therefore, at 4 h after staurosporine addition, as shown in Figure 8. Staurosporine induced the highest caspase activities, with the order: caspase-3 > caspase-6 > caspase-8 = caspase-9. All four caspases were susceptible to both antisense treatment and CSA, but the degree of inhibition (20–48 %) was less than following the other stimuli.

DISCUSSION

CyPs catalyse the interconversion of *cis* and *trans* isomers of peptidylprolyl bonds in protein substrates (for reviews see [28,29]). The PPIase activity of CyP is blocked by CSA, and CSA is frequently used to probe CyP function *in vivo* and the involvement of CyP in pathogenetic mechanisms. Accordingly, the neuroprotective capacity of CSA has been widely tested and it is now established that CSA can drastically reduce both glutamate-induced death of cultured neurons [30–32] and brain injury following transient ischaemia [14,17,33]. The therapeutic potential of CSA in this regard is currently compromised by its secondary toxic effects and poor diffusibility across the blood–brain barrier [33]. Nevertheless, the fact that CSA is protective indicates that a CSA-binding protein plays an important role in some aspect of excitotoxicity. The identity of the CSA-binding protein is unresolved. Human cells contain at least twelve CyPs and CyP domain proteins [34] and, in principle, CSA may protect by interacting with any of these. In the present study we have used an antisense ODN to examine specifically the role of CyP-A. To do this, it was first necessary to establish a working system for excitotoxin-induced death of these cells.

The study revealed a highly synergistic dependence of B50 cell death on both exogenous glutamate and NO. The dual requirement contrasts with the vulnerability of most other neuronal types in which glutamate alone is toxic [1,4,35]. There are strong indications, however, that even in these cases, NO is required and that it is supplied endogenously during glutamate stimulation. Glutamate toxicity is rooted in excessive stimulation of NMDA receptors [1]. Sattler and coworkers [36] have shown that the scaffolding protein PSD-95, responsible for clustering NMDA receptors in the plasma membrane, can also recruit neuronal

nitric oxide synthase (nNOS), a Ca^{2+}-dependent enzyme. The formation of an NMDA receptor–PSD-95–nNOS complex would be expected to produce Ca^{2+} microdomains around nNOS during receptor stimulation, thereby providing a mechanism for endogenous NO production during glutamate toxicity. Various lines of evidence indicate that NO production is essential for excitotoxicity. Glutamate toxicity, for example, depends on influx specifically via NMDA receptors [37], and cellular Ca^{2+} overload in itself (e.g. via kainate receptors [38]) is relatively innocuous unless supplemented by an exogenous supply of NO [39]. Conversely, glutamate toxicity is markedly attenuated by inhibitors of nNOS [5], and neurons from nNOS-deficient mice are resistant to glutamate toxicity [3]. The overt need for NO in B50 cells may be due to any of a number of possible causes. Glutamate induces a small rise in cytosolic Ca^{2+} in B50 cells (calcium measurements using fluo-3 as indicator; M. Capano, unpublished work), and it will be interesting to learn the subtype of receptor involved and whether it is linked to NO production or whether the Ca^{2+} influx is insufficient to elicit significant NO production. The essential point to emerge, however, is the acute synergism between glutamate and NO. This not only underlines the necessity of NO but also shows that glutamate toxicity is not mediated wholly via NO production. This is most evident at 10 μM nitroprusside, where both glutamate and nitroprusside are required. B50 cells may provide a useful system for studying Ca^{2+}/NO synergism in excitotoxicity.

The second outcome of this work is the finding that glutamate/NO engages the apoptotic pathway less effectively than oxidative stress and yields a different profile of caspase activation. Thus caspase-3 was 10-fold more active than caspase-6 under oxidative stress (Figure 7) and about 3-fold more active under glutamate/NO (Figures 4 and 6). This difference is perhaps surprising since oxidative stress is considered to be a key element in excitotoxicity. Thus stimulation of NMDA receptors enhances production of superoxide [9,10], and overexpression of superoxide dismutase increases resistance to glutamate toxicity [40]. By extension, administration of t-butylhydroperoxide, which increases both reactive oxygen species and B50 cell Ca^{2+} (M. Capano, unpublished results), might be expected to reproduce an excitotoxic profile of caspase activation but this was not the case. In some neuronal types, the action of the Ca^{2+}-dependent protease calpain is believed to compromise caspase processing [2]. However, the rise in cytosolic Ca^{2+} in B50 cells induced by glutamate/NO is rather less than that brought about by 100 μM peroxide (unpublished results), yet peroxide induces massive activation of caspase-3 (Figure 7). On these grounds, it seems unlikely that the relatively poor activation of caspase-3 in B50 cells after glutamate/NO exposure is a consequence of Ca^{2+}-activation of calpain.

We were unable to detect DNA cleavage following glutamate/NO treatment, despite the fact that it was quite evident after peroxide and staurosporine treatment. The DNA cleavage, evident as DNA laddering on agarose gels (Figure 2B), is catalysed by caspase-activated DNase (CAD). In non-apoptosing cells, CAD exists as an inactive complex with the inhibitor subunit, ICAD [41]. Caspase-3 brings about CAD activation by cleaving ICAD, resulting in release of the catalytic subunit. In a survey to determine which of the caspases were able to cleave ICAD, Wolf and co-workers [42] found that caspase-3, and to a much lesser extent caspase-7, were the only caspases with this activity. Thus the absence of detectable DNA cleavage during glutamate/NO toxicity in B50 cells may well be a consequence of the relatively poor activation of caspase-3. From this it follows that cells entering the apoptotic pathway during excitotoxic stimuli may eventually die without the 'normal' complement of

apoptotic characteristics. The biochemical and morphological profile of such cells will be defined, in part, by their particular profile of caspase processing.

The third, and major, outcome of this study is the discovery that CyP-A has a role in caspase activation. It is important to distinguish between this role and the potential capacity of CyP-A to mediate CSA protection by forming a CyP-A–CSA complex inhibitory to calcineurin. In this study we provide direct evidence that CyP-A is involved in apoptosis and that CSA blocks caspase activation by inhibiting the PPIase activity of CyP-A. This was established by suppressing CyP-A expression with the antisense ODN. It seems that CyP-A is turned over relatively quickly in B50 cells since antisense inhibition of translation resulted in $> 90\%$ loss of CyP-A within 24 h. CyP-A suppression was almost as effective as its inhibitor, CSA, in preventing caspase activation. Both CyP-A suppression and CSA exerted their strongest inhibitory effects on the activations of caspase-8, -9 and -6, and the weakest on activation of caspase-3. The locus of CyP-A action remains to be established. CyPs can form functional complexes with other proteins. For example, recognised CyP complexes include the heat shock protein Hsp90 and the antioxidant protein Aop1 (with CyP-A [43]), the oestrogen receptor (with CyP-40 [44]), the nuclear pore complex (with Nup358, a CyP-domain protein [45]), and the mitochondrial voltage-dependent anion channel-ANT complex (with CyP-D, [46]). On these grounds, CyP-A most probably forms a critical complex with some protein(s) involved in caspase processing. The fact that CyP-A suppression also inhibited caspase activation by staurosporine, albeit to a lesser extent, indicates that CyP-A is involved in caspase processing itself rather than the pathological events that trigger caspase activation.

Finally, it is important to stress that cells contain a number of CyPs, and the present finding of a role for CyP-A in excitotoxin-induced apoptosis in no way excludes additional roles for other CyP isoforms. In particular, CSA blocks the PT pore, which is believed to be involved in the pathogenesis of necrotic and apoptotic cell death in association with cellular Ca^{2+} overload and oxidative stress in a range of tissues (reviewed in [12,14,47,48]). Also, the CyP-CSA complex inhibits the Ca^{2+}-calmodulin activated protein phosphatase calcineurin, which can dephosphorylate numerous intracellular proteins. Calcineurin is also inhibited by the complex of the drug FK506 and its binding protein FKBP. The fact that in some systems, at least, both FK506 and CSA reduce glutamate toxicity suggests that calcineurin inhibition may also lead to neuroprotection [31]. Neither of these possibilities is excluded by the present study, since they were not tested.

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