Mitochondrial apoptotic cell death and moderate superoxide generation upon selective activation of non-desensitizing AMPA receptors in hippocampal cultures

A. Cristina Rego, Nuno M. Monteiro, Ana P. Silva, Joana Gil, João O. Malva and Catarina R. Oliveira

Institute of Biochemistry, Faculty of Medicine and Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

Abstract

In the present work we investigated the effect of selective stimulation of non-desensitizing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors in the intracellular processes leading to hippocampal neuronal death and production of reactive oxygen species (ROS). Activation of AMPA receptors in the presence of cyclothiazide (CYZ), a blocker of AMPA receptor desensitization, resulted in the death of approximately 25% of neurones, which was prevented by 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(f)quinoxaline (NBQX), an AMPA-preferring receptor antagonist. (+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801) protected the neurones from necrotic death induced by AMPA or NMDA receptor activation. Neurodegeneration caused by selective activation of non-desensitizing AMPA receptors, in the presence of AMPA, CYZ and MK-801, significantly decreased the number of Co^{2+}-positive neurones, used as a cytochemical marker of Ca^{2+}-permeable AMPA receptors, but maintained intracellular ATP/ADP. The AMPA-mediated apoptotic cell death involved mitochondrial cytochrome *c* release and the activation of caspases-1 and -3, which was prevented by NBQX. Interestingly, although selective activation of AMPA receptors was not associated with production of intracellular peroxides, a moderate increase in superoxide production was observed upon exposure to antimycin A (AA). Furthermore, increased activity of Mn-superoxide dismutase (SOD) was observed on selective activation of non-desensitizing AMPA receptors. Taken together, these data make important contributions to the elucidation of the downstream pathways activated in AMPA receptor-mediated excitotoxicity in cultured rat hippocampal neurones.

Keywords: AMPA receptors, caspases, cytochrome *c*, hippocampal neurones, neurotoxicity, reactive oxygen species.

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Activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors produces fast and rapidly desensitizing responses, which can be blocked by specific drugs, such as cyclothiazide (CYZ), a preferential blocker of the AMPA receptor subtype (Partin *et al*. 1993). This blockade is associated with a higher toxicity, because the receptors stay open for longer periods and may be permeable to Ca^{2+}, as shown in hippocampal or cortical neurones (May and Robinson 1993; Cebers and Liljequist 1995; Ohno *et al*. 1998), although populations of hippocampal neurones have shown distinct sensitivities to AMPA, kainate or CYZ in terms of neuronal viability and intracellular Ca^{2+} changes (Ambrósio *et al*. 2000). AMPA receptors assemble from the combination of GluR1 to GluR4 subunits, but the presence of GluR2 subunits, whose expression is regulated in a cell-specific manner, greatly reduces Ca^{2+} permeability,

voltage-dependent block by polyamines and single-channel conductance (Pellegrini-Giampietro *et al.* 1997; Tanaka *et al.* 2000 for review). Under physiological conditions, excitatory neurones in the hippocampus express abundant GluR2 mRNA, and exhibit low Ca^{2+} permeability (Geiger *et al.* 1995). Therefore, a decreased expression of GluR2 subunits has been implicated in neuronal vulnerability, leading to cell death under ischaemic conditions (Gorter *et al.* 1997; Opitz *et al.* 2000; Tanaka *et al.* 2000).

Activation of AMPA receptors is associated with intracellular Ca^{2+} accumulation, mitochondrial depolarization (Rego *et al.* 2001) and production of reactive oxygen species (ROS), namely the superoxide anion (e.g. Carriedo *et al.* 1998). Mitochondrial dysfunction and swelling have been associated with the release of cytochrome c into the cytoplasm, namely through the opening of the permeability transition pore, implicating the mitochondria as an important site in the regulation of cell death. Within the cytosol, cytochrome c interacts with Apaf-1 in the presence of dATP, activating caspase-9, which further activates caspase-3 and may result in apoptosis mediated by AMPA receptors. The neurotoxic effects of AMPA/kainate receptor stimulation, documented through the involvement of neuronal Na^+ loading on cytosolic Ca^{2+} changes and neuronal swelling (Itoh *et al.* 1998; Kiedrowski 1998; Rego *et al.* 2001), have been associated with both necrotic and/or apoptotic types of cell death, as reported in cerebellar granule cells (Simonian *et al.* 1996; Cebers *et al.* 1997) or cortical neurones (Larm *et al.* 1997). Moreover, blockade of AMPA receptor desensitization with CYZ was shown to modulate the receptor-mediated apoptosis in cortical neuronal cultures, as determined by monitoring cell viability and morphology (John *et al.* 1999). However, less is known about the influence of selective activation of AMPA receptors under non-desensitizing conditions on apoptotic features of cell death, such as cytochrome c release or caspase activation, and their correlation with intracellular generation of ROS in hippocampal neurones.

In the present work we therefore investigated cell death after stimulation of desensitizing or non-desensitizing AMPA receptors in hippocampal neuronal cultures. Particular attention was given to the analysis of selective activation of AMPA receptors under non-desensitizing conditions, in the presence of CYZ (a preferential blocker of AMPA receptor desensitization) and (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801), the non-competitive inhibitor of NMDA receptors. Under these conditions, we show that brief stimulation of AMPA receptors induces hippocampal neuronal death by necrosis and apoptosis, release of cytochrome c, activation of caspases and DNA condensation/fragmentation (Monteiro *et al.* 2001). Concomitantly, a moderate generation of mitochondrial superoxide was counterbalanced by an increased activity of Mn-superoxide dismutase (SOD), although this did not involve the formation of intracellular peroxides (Monteiro

et al. 2001). Because the hippocampus is involved in neurodegeneration caused by epileptic seizures, ischaemia or Alzheimer's disease, the increased sensitivity of hippocampal neurones to excitotoxicity mediated by selective AMPA receptor stimulation may be relevant in defining the vulnerability of neurones in this elective cerebral area.

Materials and methods

Materials
Neurobasal medium, B27 supplement, gentamicin and trypsin were from Gibco-BRL, Life Technologies (Paisley, Scotland, UK). AMPA, 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(f)quinoxaline (NBQX) and CYZ were obtained from Tocris Cookson (Bristol, UK). Mitotracker-green, syto-13, propidium iodide (PI), anti-fade kit, Alexa Fluor-594 anti-mouse IgG and Alexa Fluor-488 anti-rabbit IgG were purchased from Molecular Probes (Leiden, The Netherlands). Antibody against the native or denatured form of cytochrome c was obtained from PharMingen (San Diego, CA, USA). Anti-active caspase-3 was obtained from Promega (Madison, WI, USA). Colorimetric substrates for caspases-1 and -3 were purchased from Calbiochem (San Diego, CA, USA). MK-801 was a kind gift from Merck Sharp & Dohme Research Laboratories (Merck & Co. Inc., Whitehouse Station, NJ, USA). All other reagents, including anti-microtubule-associated protein (MAP)-2 and caspase-8 substrate, were obtained from Sigma Chemical (St Louis, MO, USA).

Culture and incubation of rat hippocampal neurones
Hippocampal neurones were obtained from 18–19-day-old Wistar rat embryos, according to the National Ethical Requirements for Animal Research and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. The cells were dissociated with trypsin (1 mg/mL) for 15 min at 37°C and deoxyribonuclease I (0.15 mg/mL) in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM $Na_2HPO_4.2H_2O$, 4.16 mM $NaHCO_3$, 5 mM glucose, 1 mM sodium pyruvate and 10 mM HEPES, pH 7.4). The cells (1×10^5 cells/cm²; plated on poly-L-lysine-pretreated 12-well dishes for lactate dehydrogenase (LDH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, or six-well dishes for measurement of enzyme activities) were cultured for 7–8 days (95% air, 5% CO_2) in serum-free Neurobasal medium, supplemented with B27 supplement (2%), glutamate (25 μM), glutamine (0.5 mM) and gentamicin (0.12 mg/mL), according to Ambrósio *et al.* (2000). For immunocytochemistry, morphological studies and analysis of ROS formation, neurones were mounted on coverslips that had been pretreated with poly-L-lysine (45×10^3 cells/cm²). These primary hippocampal neuronal cultures were shown to contain approximately 95% neurones, as determined by immunocytochemistry with anti-MAP-2 antibody and anti-glial fibrillary acidic protein. As demonstrated previously (Silva *et al.* 2001) MAP-2 was shown to be localized throughout the neurones, particularly in the dendrites.

Hippocampal neurones were exposed to AMPA (100 μM), in the absence or presence of CYZ (30 μM) and MK-801 (10 μM) for 15 min, in supplemented Neurobasal medium. The cells were then

washed twice with saline buffer (132 mM NaCl, 4 mM KCl, 1.4 mM MgCl$_2$, 1 mM CaCl$_2$, 6 mM glucose and 10 mM HEPES-Na, pH 7.4) and incubated for 24 h in supplemented Neurobasal medium. Some neurones were also incubated with NBQX (20 µM), an AMPA-preferring receptor antagonist. Where indicated, the neurones were exposed to NMDA (100 µM) and glycine (10 µM), in the presence of NBQX (20 µM), in Mg^{2+}-free medium.

Analysis of neuronal death

The integrity of the plasma membrane of hippocampal neurones was monitored by the leakage of LDH, by following the rate of conversion of NADH to NAD$^+$ at 340 nm, according to Bergmeyer and Bernt (1974). LDH released into the extracellular medium was expressed as a percentage of total LDH activity in the cells. Cell viability at 37°C was also measured by following the reduction of MTT at 570 nm (Mosmann 1983).

Analysis of neuronal death by apoptosis or necrosis was assessed using the fluorescent probes syto-13, a membrane-permeable green fluorescent dye that binds DNA and RNA, and PI, a non-membrane-permeable red fluorescent dye that binds to DNA. Viable cells show a green nucleus labelled homogenously with syto-13, whereas apoptotic cells show condensed and fragmented nuclei labelled with syto-13 (primary apoptosis) or with syto-13 plus PI (secondary apoptosis). Necrotic neurones are visualized by an intense red fluorescence upon labelling with PI. The neurones were loaded for 3 min with a solution of saline buffer containing 4 µM syto-13 and 4 µg/mL PI. Viable, apoptotic or necrotic cells were counted by using an inverted Nikon Diaphot-TMD fluorescence microscope (Nikon Corporation, Tokyo, Japan), with a triple filter Omega XF63 (Omega Optical Inc., Brattleboro, VT, USA).

Identification of Ca^{2+}-permeable AMPA receptors

After incubation for 24 h, the cells were washed twice in HEPES buffer containing 146 mM NaCl, 4.2 mM KCl, 0.5 mM MgCl$_2$, 0.8 mM CaCl$_2$, 20 mM HEPES and 55.6 mM glucose, at pH 7.4, and stimulated with AMPA plus CYZ and MK-801 in HEPES buffer containing 5 mM CoCl$_2$. The cells were washed twice in HEPES buffer containing 2 mM EDTA then cobalt was precipitated with 0.12% Na$_2$S for 5 min. The cells were fixed with 4% paraformaldehyde containing 4% sucrose in phosphate-buffered saline (PBS) for 30 min, followed by 1 h incubation at 50°C with 1 mg/mL AgNO$_3$ in developing buffer (292 mM sucrose, 15.5 mM hydroquinone, 42 mM citric acid). The neurones were also visualized with anti-MAP-2 (1 : 500), after cell permeabilization with Triton X-100 (0.2%). The neurones were visualized in an inverted Nikon Diaphot fluorescence microscope, with a triple filter Omega FX-64, and counted from arbitrary fields in the culture.

Measurement of adenine nucleotides

ATP and ADP were analysed after cell extraction with 0.3 M perchloric acid (0–4°C). The cells were centrifuged at 15 800 *g* for 5 min, and the pellet was solubilized with 0.1 M NaOH for total protein analysis by the Biorad method (Bradford 1976). The supernatants were neutralized with 3.3 M KOH in 1.67 M Tris and further centrifuged at 15800 *g* for 10 min. The supernatants were then stored at − 80°C. They were assayed for adenine nucleotides by reverse-phase HPLC, using a mobile phase of 10 mM NaH$_2$PO$_4$, pH 6.0, and 14% methanol, at a flow rate of 1.5 mL/min.

Immunocytochemistry

For analysis of cytochrome *c*, the cells were washed three times with saline buffer and incubated with mitotracker-green (500 nM), a mitochondrial marker that is insensitive to the mitochondrial potential (Krohn *et al.* 1999). After fixation in 4% paraformaldehyde containing 4% sucrose in PBS for 10 min, the cells were washed three times with PBS and incubated with 20 mM glycine. After cell permeabilization in the presence of saponin (0.1%), the cells were incubated with a specific antibody against the native form of cytochrome *c* (1 : 100), for 30 min. For analysis of caspase-3, the cells were also fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton (in PBS) for 5 min, washed three times in PBS, and further incubated with a blocking buffer containing 3% BSA in PBS/0.1% Tween for 2 h. The cells were incubated with anti-active caspase-3 (1 : 250) and anti-MAP-2 (1 : 500), overnight at 4°C. Cytochrome *c* and MAP-2 were visualized by using the secondary antibody Alexa Fluor-594 anti-mouse IgG (1 : 200), whereas active caspase-3 was visualized by using the secondary antibody Alexa Fluor-488 anti-rabbit IgG (1 : 200). The cells were visualized by confocal microscopy (MRC 600; Bio-Rad, Hercules, CA, USA).

Western blot analysis of cytochrome *c*

The cells were extracted in buffer containing 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM EGTA, pH 7.4, supplemented with 1 mM dithiothreitol, 100 µM phenylmethylsulphonyl fluoride (PMSF) and protease inhibitor cocktail (1 : 100; Sigma), and centrifuged at 500 *g* for 12 min at 4°C. The mitochondrial fraction was obtained by centrifugation at 12000 *g* for 20 min. The proteins were separated by electrophoresis on 15% sodium dodecyl sulfate polyacrylamide gels and transferred to a polyvinylidene difluoride membrane. The membrane was further blocked with 5% fat-free milk before incubation with a specific antibody against the denatured form of cytochrome *c* (1 : 1000). An anti-mouse IgG secondary antibody (1 : 25 000) was used (ECF system; Amersham, UK) and visualized by scanning on a STORM 860 (Molecular Dynamics, Sunnyvale, CA, USA), using a blue excited fluorescence.

Caspase activity assay

The cells were rinsed twice in PBS and then extracted with a lysis buffer (1 mM sodium EDTA, 1 mM sodium EGTA, 2 mM MgCl$_2$, 25 mM HEPES, pH 7.5) containing 2 mM dithiothreitol, 0.1 mM PMSF and protease cocktail inhibitors (1 : 100; Sigma). The samples were further centrifuged at 25800 *g* for 10 min at 4°C. The resulting supernatants (equivalent to 25 µg protein, as determined by the Biorad method) were used to determine the activity of caspases, at 405 nm, in buffer containing 10% sucrose, 1% 3[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS), 25 mM HEPES, pH 7.4, 10 mM dithiothreitol, and 100 µM of the colorimetric substrates *N*-acetyl-Tyr-Val-Ala-Asp-*p*-nitroanilide (Ac-YVAD-pNA), *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-pNA) or *N*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide (Ac-IETD-pNA) for measurement of caspase-1, caspase-3 and caspase-8-like activities respectively.

Measurement of ROS generation

Intracellular peroxides were measured by following the oxidation of dichlorodihydrofluorescein diacetate (DCFH$_2$-DA; 20 µM) or dihydrorhodamine-123 (DHR-123; 5 µM) to the fluorescent DCF or

rhodamine-123 respectively, according to Royall and Ischiropoulos (1993) and Rego *et al*. (1999). The neurones were loaded with DCFH$_2$-DA for 20 min in saline buffer at 37°C, in the dark. The increase in DCF fluorescence was measured in saline buffer (37°C), with excitation at 502 nm and emission at 550 nm. The increase in fluorescence of DHR-123 was measured in saline buffer (37°C), with excitation at 500 nm and emission at 536 nm. Loading of the probes was renewed for each experiment.

Superoxide generation was determined after incubation with 5 μM hydroethidine for 60 min at 37°C (the probe was also renewed for each experiment), according to the method described in single cells by Bindokas *et al*. (1996) or Budd *et al*. (1997). The fluorescence of ethidium, obtained after oxidation of hydroethidine (dihydroethidium) by superoxide was measured in the cuvette for 20 min at 37°C, with excitation at 548 nm and emission at 620 nm. A SPEX Fluorolog spectrometer (Industries Inc., Edison, NJ, USA) equipped with a temperature-controlled water bath was used in these experiments.

Measurement of specific SOD activity

To determine the activity of SOD (total and Mn-SOD), the hippocampal neurones were extracted with 0.5% NP-40, according to the method described by Troy and Shelanski (1994) and Troy *et al*. (2000). Briefly, the NP-40 cellular extracts were incubated in buffer containing 50 mM NaHCO$_3$, 0.1 mM EDTA, pH 10.2, plus 0.03% Triton X-100, 100 μM xanthine and 0.025 U/mL xanthine oxidase. The reduction of nitroblue tetrazolium (25 μM) was measured at 560 nm in the absence or presence of 1 mM potassium cyanide (KCN), to discriminate Cu/Zn-SOD from Mn-SOD. Total SOD activity was measured in the absence of KCN, whereas Mn-SOD activity was measured in the presence of KCN, which inhibits Cu/Zn-SOD.

Data analysis

Data are the mean ± SEM from at least three experiments, performed in duplicate or triplicate, as indicated in figure legends. Statistical analysis was performed by unpaired student's *t*-test or by one-way ANOVA, followed by Tukey *post-hoc* test; $p < 0.05$ was considered significant.

Results

Selective activation of AMPA receptors mediates hippocampal neuronal death by apoptosis and necrosis

Changes in neuronal viability associated with the stimulation of AMPA receptors under desensitizing or non-desensitizing conditions were analysed by the MTT assay (Fig. 1a) and the release of LDH (Fig. 1b), which demonstrate the cell-reducing capacity and the disruption of the plasma membrane respectively. Activation of AMPA receptors under non-desensitizing conditions in the presence of AMPA (100 μM) plus CYZ (30 μM), a preferential blocker of AMPA receptor desensitization, was associated with a significant decrease in cell viability (by about 25%). In previous studies, 30 μM CYZ was shown to maximally decrease the ability of hippocampal neurones to reduce MTT (A. F. Ambrósio,
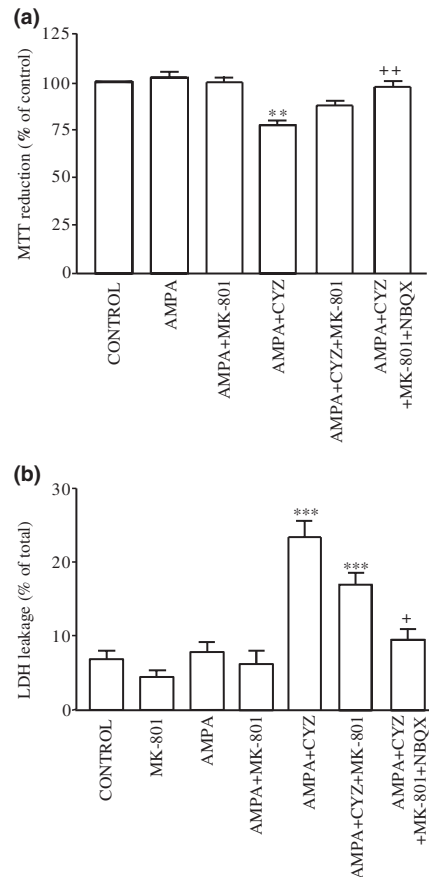
Fig. 1 MTT reduction or LDH leakage upon selective activation of non-desensitizing AMPA receptors. Hippocampal neurones were exposed to 100 μM AMPA with or without 30 μM CYZ and 10 μM MK-801, and further evaluated for changes in cell viability by the MTT test (a) or by the release of LDH (b). NBQX (20 μM) prevented the decrease in MTT reduction (a) or LDH leakage (b) induced by AMPA + CYZ in the presence of MK-801. Values are mean ± SEM of three to 14 experiments in triplicate. **$p < 0.01$, ***$p < 0.001$ versus control; +$p < 0.05$ and ++$p < 0.01$ versus AMPA + CYZ + MK-801-treated cells (one-way ANOVA followed by Tukey *post-hoc* test).

unpublished results). Incubation with MK-801 (Figs 1a and b), to counteract the effect of secondary NMDA receptor activation (Itoh *et al*. 1998), partially prevented the neuronal death observed following treatment with AMPA plus CYZ. Substantial prevention of cell death induced by AMPA in the presence of CYZ was observed after incubation with MK-801 and NBQX, antagonists of NMDA and AMPA receptors respectively (Figs 1a and b). Nevertheless, the alterations in cell viability between the distinct experimental conditions could not be attributed to significant changes in intracellular ATP/ADP levels (Table 1).

Features of apoptotic, necrotic or viable cells were examined by counting the number of hippocampal neurones labelled with the fluorescent probes syto-13 (membrane permeable) and PI (membrane impermeable), a method used

Table 1 Intracellular levels of ATP/ADP in hippocampal neurones incubated with AMPA, under desensitizing or non-desensitizing conditions

	ATP/ADP
Control	12.89 ± 0.86
MK-801	15.44 ± 2.07
AMPA	15.76 ± 1.30
AMPA + MK-801	15.09 ± 0.98
AMPA + CYZ	16.17 ± 2.68
AMPA + CYZ + MK-801	13.54 ± 1.09
AMPA + CYZ + MK-801 + NBQX	13.28 ± 0.49

Data are the mean ± SEM of three to six experiments, run in triplicate. Hippocampal neurones were incubated with 100 μM AMPA, in the absence or in the presence of CYZ (30 μM), MK-801 (10 μM) or NBQX (20 μM) for 15 min, and further incubated in Neurobasal medium for 24 h, in the absence of the drugs. The levels of ATP/ADP were determined by reverse-phase HPLC after perchloric extraction. No statistical differences were observed.

to analyse chromatin condensation and/or fragmentation (Figs 2 and 3). Compared with cells incubated under control conditions, which showed some apoptotic features (about 10% of total cells), activation of AMPA receptors under desensitizing conditions (AMPA alone) induced a significant increase in the percentage of cells (approximately 10%) undergoing necrosis, which was completely prevented by MK-801 (Fig. 2). This small percentage of cells undergoing necrosis upon incubation with AMPA (Fig. 2) was not observed when measuring the changes in hippocampal neuronal viability (Figs 1a and b). Selective activation of AMPA receptors under non-desensitizing conditions induced by CYZ, and in the presence of MK-801, was associated with both apoptotic and necrotic cell death. Furthermore, in the presence of AMPA plus CYZ, MK-801 was shown to significantly decrease the number of necrotic neurones (by about 5% compared with AMPA/CYZ alone; $p < 0.05$), despite maintenance of the number of viable neurones and a non-significant increase (about 5%) in the number of apoptotic neurones (Fig. 2). A decrease in the number of neurones entering necrosis upon exposure to MK-801, compared with AMPA/CYZ only (Fig. 2), was reflected by a decrement in LDH release under the same experimental conditions (Fig. 1b), suggesting the prevention of plasma membrane disruption and, consequently, a decrease in the number of neurones undergoing necrosis. In addition, hippocampal neuronal death was completely prevented upon blockade of both NMDA and AMPA receptors, respectively, in the presence of MK-801 and NBQX (Fig. 2), similar to data in Fig. 1. Incubation with MK-801 (Figs 1b and 2) or CYZ (not shown) alone did not significantly affect the percentage of viable, apoptotic or necrotic neurones, compared with the control.
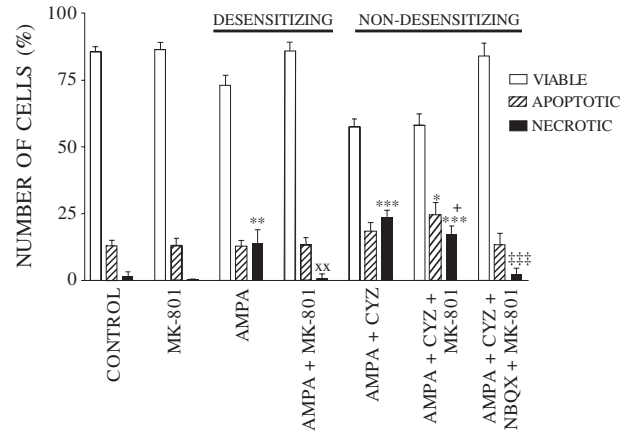
Fig. 2 Cell death by necrosis or apoptosis induced by activation of AMPA receptors, under desensitizing or non-desensitizing conditions. Hippocampal neurones were incubated with 100 μM AMPA, in the absence (desensitizing) or in the presence of 30 μM CYZ (non-desensitizing). MK-801 (10 μM), a non-competitive antagonist of NMDA receptors, was further tested. Necrotic and apoptotic neuronal death, determined with syto-13 and PI, was prevented upon addition of 20 μM NBQX, an AMPA-preferring receptor antagonist, in neurones exposed to AMPA + CYZ + MK-801. Values are mean ± SEM of five to 13 experiments. A total of about 200 cells per coverslip was counted, from three coverslips per experiment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus respective control; +$p < 0.05$ versus AMPA + CYZ-treated cells; ‡‡‡$p < 0.001$ versus AMPA + CYZ + MK-801-treated cells; xx$p < 0.01$ versus AMPA-treated cells (one-way ANOVA followed by Tukey *post-hoc* test).
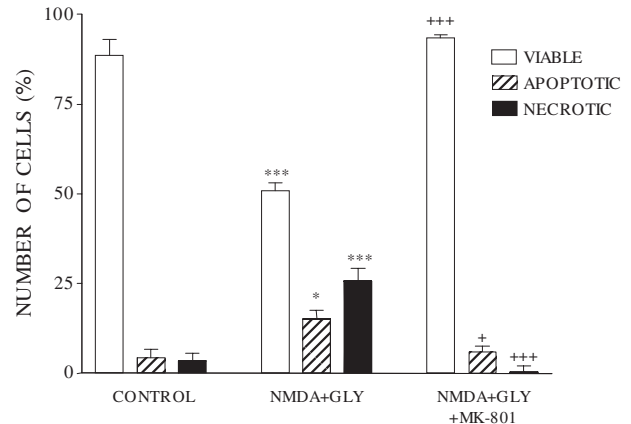

Fig. 3 Selective NMDA-induced hippocampal neuronal death. The neurones were incubated with 100 μM NMDA plus 10 μM glycine (GLY), in the presence of NBQX (20 μM) in Mg^{2+}-free medium. Neuronal death, determined with syto-13 and PI, was almost completely abolished upon addition of 10 μM MK-801, an NMDA receptor antagonist. Values are mean ± SEM of three to four experiments. A total of about 200 cells per coverslip was counted, from three coverslips per experiment. *$p < 0.05$, ***$p < 0.001$ versus control; +$p < 0.05$, +++$p < 0.001$ versus NMDA + GLY-treated cells (one-way ANOVA followed by Tukey *post-hoc* test).

In order to evaluate the role of NMDA receptor activation in hippocampal neuronal death, we examined the effect of NMDA receptor stimulation upon exposure to NMDA plus glycine in Mg^{2+}-free medium. The medium also contained NBQX to eliminate the effect of AMPA receptors (Fig. 3). Under these conditions, the number of viable cells was greatly decreased, concomitant with an increase in the percentage of apoptotic (about 15%) and, in particular, necrotic (about 25%) cells. Furthermore, specific blockade of NMDA receptors with MK-801 reduced the numbers of both apoptotic and necrotic cells and enhanced the percentage of viable cells, similar to control conditions (Fig. 3). These results show that apoptosis and necrosis are common features induced by selective activation of both AMPA and NMDA receptors. Furthermore, subsequent NMDA receptor activation enhanced the number of necrotic neurones upon stimulation of AMPA receptors.

To determine whether hippocampal neuronal death was associated with neurones containing Ca^{2+}-permeable AMPA receptors, the neurones (control or AMPA/CYZ treated) were subjected to secondary activation of AMPA receptors under non-desensitizing conditions, in the presence of MK-801 and Co^{2+}, a procedure known to enhance intracellular Co^{2+} labelling associated with the presence of Ca^{2+}-permeable AMPA receptors (Malva *et al*. 2003). Figure 4(a–d) shows that, in the population of hippocampal neurones visualized with the neuronal marker MAP-2, only some control neurones were stained with Co^{2+}, indicating that a small percentage of cells contained Ca^{2+}-permeable AMPA receptors (about 15–20%). Furthermore, we observed a significant decrease ($\sim$25% of control) in Co^{2+}-stained neurones after non-desensitizing activation of AMPA receptors (Fig. 4c–e), suggesting that the neurones containing Ca^{2+}-permeable AMPA receptors were partially sensitive to the excitotoxic insult.

CONTROL + MK-801

AMPA + CYZ + MK-801

Co^{2+} staining anti-MAP2

Fig. 4 Co^{2+} staining after selective activation of AMPA receptors in the presence of CYZ. Hippocampal neurones were incubated with 100 μM AMPA in the presence of 30 μM CYZ (non-desensitizing conditions) and 10 μM MK-801 (c,d), and compared with control neurones incubated with MK-801 only (a,b). After 24 h incubation in Neurobasal medium, the neurones (control and AMPA/CYZ-treated) were stimulated with AMPA + CYZ + MK-801, in the presence of Co^{2+}. Co^{2+}-stained neurones were counted from an arbitrary field in the culture, and compared with anti-MAP-2 staining (red). The arrows, in Co^{2+}-stained neurones, but not those stained for MAP-2 (b,d), show hippocampal neurones containing Ca^{2+}-permeable AMPA receptors. Note that (a) or (b) (control) and (c) or (d) (AMPA/CYZ) are representative images from four independent experiments. (e) Changes in Co^{2+} staining over the total MAP-2 staining of AMPA + CYZ + MK-801-treated hippocampal neurones compared with the control (in the presence of MK-801). Values are mean ± SEM from four experiments run in triplicate. A total of about 200 cells per coverslip were counted, from three coverslips per experiment. *$p < 0.05$ versus control (unpaired student's *t*-test).
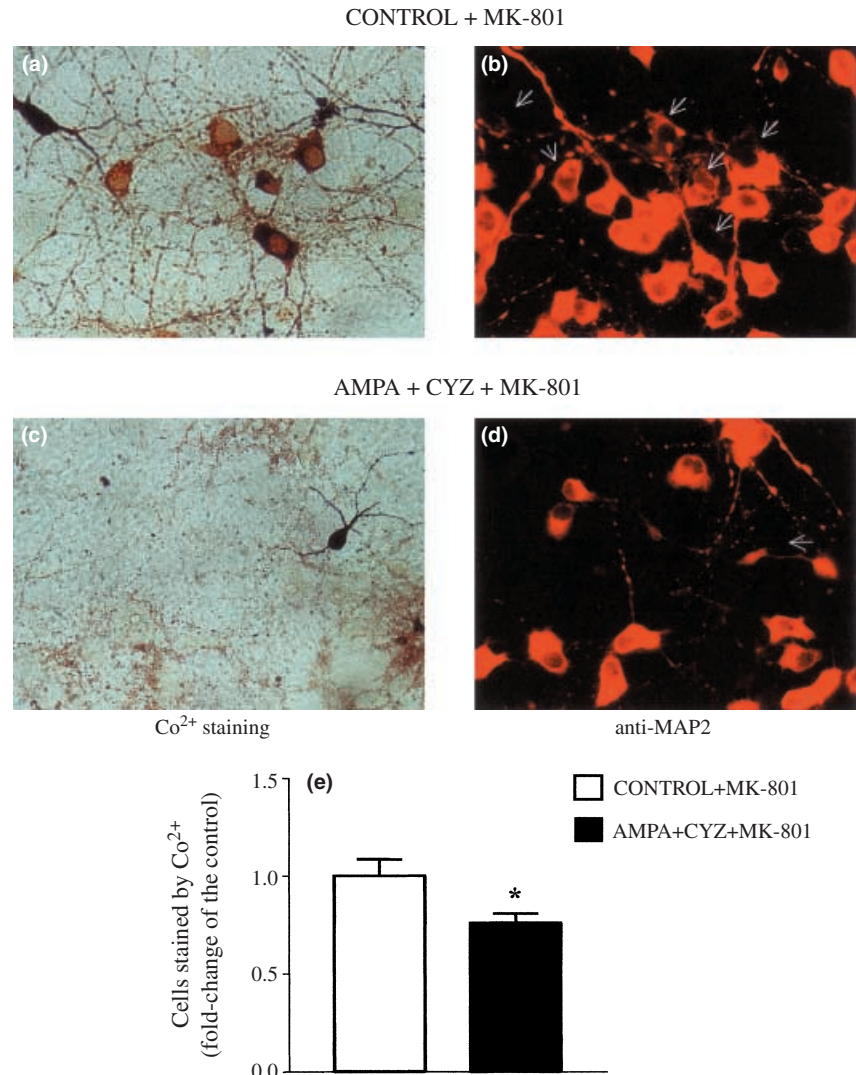
Selective stimulation of AMPA receptors under non-desensitizing conditions induces mitochondrial cytochrome *c* release and activation of caspases

In order to examine whether the features of cell death induced by selective activation of AMPA receptors under non-desensitizing conditions (in the presence of CYZ and MK-801) involved mitochondria-induced release of apoptotic factors, we analysed the release of mitochondrial cytochrome *c* and the activation of caspases.

Figure 5 shows a decrease in the number of cytochrome *c*-like immunoreactive neurones on incubation with AMPA plus CYZ (Fig. 5b), compared with the control (Fig. 5a), as determined by a decrease in the co-localization of cytochrome *c* with the mitochondria. These results were confirmed by western blotting, using an antibody against the denatured form of cytochrome *c*, and evaluated as a percentage of cytochrome *c* retained within the organelle (Fig. 5d). These results were shown to be receptor dependent, because NBQX (20 μM) prevented the decrease in mitochondrial retention of cytochrome *c* observed on exposure to AMPA, CYZ and MK-801 (Figs 5c and d).

Because activation of caspases occurs subsequently to mitochondrial cytochrome *c* release, we also determined the activation of caspases-1, -3 and -8 by immunocytochemistry or with colorimetric substrates (Fig. 6). Interestingly, in hippocampal neurones exposed to AMPA plus CYZ and MK-801 a predominant localization of active caspase-3 was observed in the nucleus (Figs 6d–f), as determined by using an antibody against its active form. Incubation with NBQX (Figs 6g–i) prevented the activation of caspase-3 induced by AMPA plus CYZ. Caspase-3 activity was confirmed by following the increase in the cleavage of Ac-DEVD-pNA (Fig. 6j). Under these conditions, caspase-8 activity was not significantly changed (Fig. 6k). Interestingly, a significant increase in Ac-YVAD-pNA cleavage, a substrate that is able to interact with caspase-1, was observed in extracts from neurones incubated with AMPA plus CYZ and MK-801 (Fig. 6l).

Selective activation of AMPA receptors predisposes hippocampal neurones to enhanced Mn-SOD activity

Because oxidative stress has been implicated in cell death by necrosis and apoptosis (Rego *et al.* 1999; Luetjens *et al.* 2000; Sugawara *et al.* 2002), we used distinct fluorescent probes to analyse the production of intracellular ROS induced by selective activation of non-desensitizing AMPA receptors, in the presence of AMPA plus CYZ and MK-801.

In order to explore the production of mitochondrial superoxide, the oxidation of hydroethidine, which generates the fluorescent ethidium, was followed in a population of control or AMPA-stimulated hippocampal neurones. Under basal conditions, no significant changes in superoxide anion formation were observed (Fig. 7). However, incubation with 5 μM antimycin A (AA), a specific inhibitor of mitochondrial

complex III, significantly enhanced ethidium fluorescence in hippocampal neurones stimulated with AMPA plus CYZ in the presence of MK-801, compared with controls (Fig. 7). A low response to AA in the population of control hippocampal neurones may be explained by the great variability of AA-induced changes in ethidium fluorescence, as observed in single-cell analysis (data not shown). The data suggest that selective activation of AMPA receptors under non-desensitizing conditions predisposes the hippocampal neurones to increased formation of mitochondrial superoxide. Under the same experimental conditions, an increase in the activity of the mitochondrial antioxidant enzyme Mn-SOD was also observed (Fig. 8), suggesting a protective effect against an increase in superoxide production. No significant changes in the protein expression levels of Mn-SOD were observed in western blot analysis (not shown). In addition, NBQX prevented the increase in Mn-SOD activity observed upon incubation with AMPA, CYZ and MK-801 (Fig. 8), providing evidence for a receptor-specific effect.

Because increased SOD activity is associated with an increase in hydrogen peroxide, we also examined the formation of intracellular peroxides, by following the oxidation of DCFH₂-DA and DHR-123 (Table 2). These probes have been reported to be oxidized by ROS, with the exception of superoxide (Royall and Ischiropoulos 1993). Table 2 shows that the formation of peroxides was not significantly different in control or AMPA/CYZ-stimulated neurones, under basal or hydrogen peroxide-stimulated conditions.

Discussion

In this work we showed that selective activation of AMPA receptors under non-desensitizing conditions induces hippocampal neuronal death by necrosis and apoptosis. Cell death by apoptosis was evaluated by the release of cytochrome *c*, the activation of caspases-1 and -3, and chromatin condensation/fragmentation, which was prevented by specific blockade of AMPA receptors. The increase in hippocampal neuronal sensitivity predisposed to a moderate generation of mitochondrial superoxide and an AMPA receptor-mediated increase in the activity of Mn-SOD, which may counterbalance the neurotoxic effects induced by the radical. However, excitotoxicity induced by AMPA could not be accounted for by a significant increase in intracellular peroxide formation, excluding the occurrence of severe oxidative stress.

In agreement with previous published evidence of cell death by apoptosis upon exposure to kainate (Campagne *et al.* 1995; Simonian *et al.* 1996), selective AMPA receptor activation (Cebers *et al.* 1997; Larm *et al.* 1997) or activation of AMPA receptors under non-desensitizing conditions (John *et al.* 1999), we also observed that selective AMPA receptor stimulation induces cell death by necrosis and apoptosis (Figs 1 and 2), although the intracellular ATP/ADP
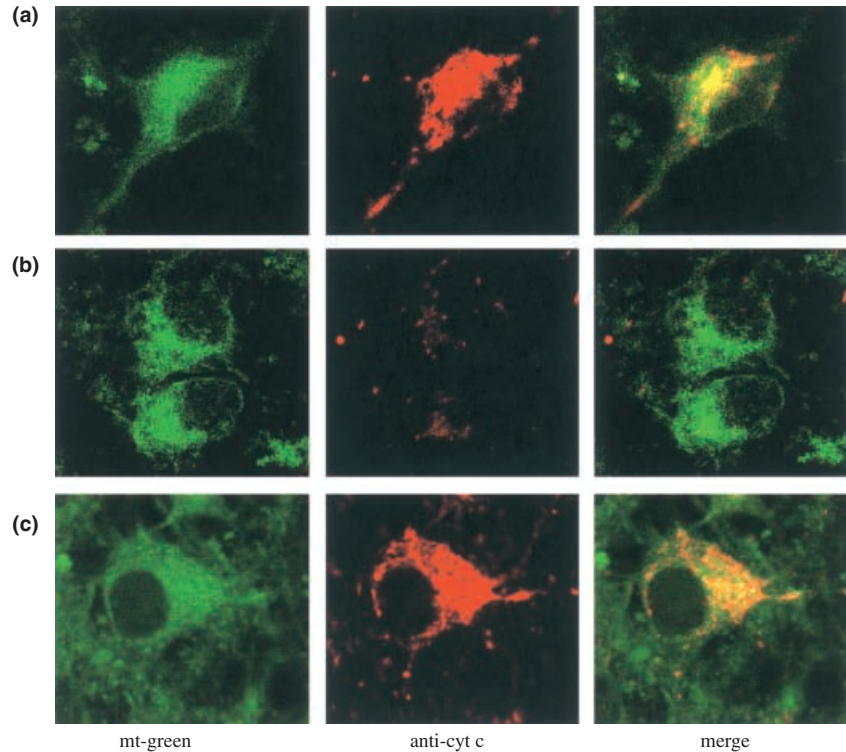
Fig. 5 Effect of selective non-desensitizing AMPA receptor activation on the release of mitochondrial cytochrome *c*. Immunocytochemistry of control (a) or 100 μM AMPA + 30 μM CYZ-treated (b) hippocampal neurones, incubated in the presence of 10 μM MK-801, show that cytochrome *c* (cyt *c*) (red) is released from the mitochondria, identified with mitotracker-green (mt-green), following the activation of AMPA receptors under non-desensitizing conditions. Addition of NBQX (20 μM) restored the co-localization of cytochrome *c* and mitotracker-green (c). Images are representative of three independent experiments. (d) The graph represents the relative amount of cytochrome *c* retained in the mitocondria (percentage of control), from four independent experiments, as determined by western blotting of mitochondria isolated from hippocampal neurones. Values are mean ± - SEM. The blot image shows a representative experiment carried out under the conditions depicted in the graph.
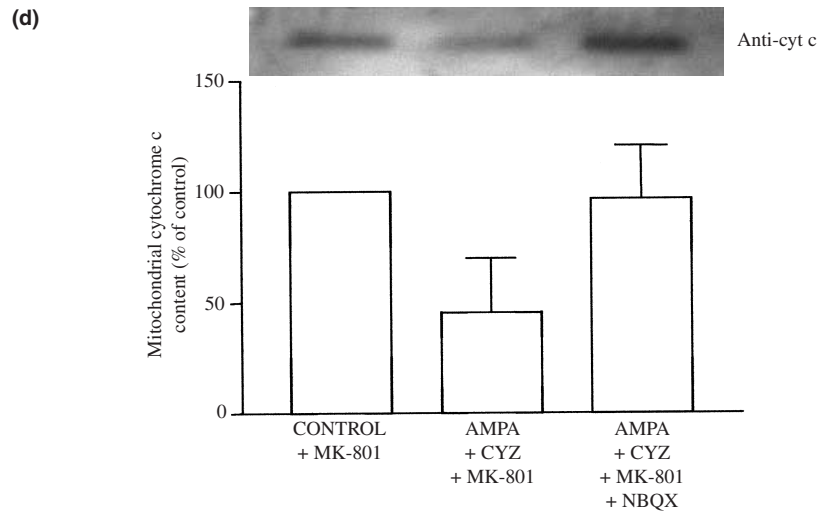
levels seemed to be preserved (Table 1). Importantly, secondary activation of NMDA receptors, which are stimulated by depolarization-induced removal of Mg^{2+} blockade, occurring after AMPA-mediated cytosolic Na^+ loading (Itoh *et al.* 1998), or selective activation of NMDA receptors, committed a higher percentage of neurones to necrosis, as observed in Figs 2 and 3. This observation should take into account that the hippocampal neurones used contain mostly NR1/NR2B receptors and that increased expression of NR2A, followed by the expression of NR3 (postnatal), mainly associated with a lower Ca^{2+} permeability, occurs later in development (Barria and Malinow 2002; Matsuda *et al.* 2002).

According to data shown in Fig. 4, the significant decrease in the number of Co^{2+}-positive neurones after selective activation of AMPA receptors suggests some susceptibility of hippocampal neurones containing Ca^{2+}-permeable AMPA receptors (Malva *et al.* 2003). These receptors are controlled by the absence of GluR2 subunits (for review, Bennett *et al.* 1996; Pellegrini-Giampietro *et al.* 1997; Brorson *et al.* 1999). In previous studies, stimulation with kainate caused a rapid influx of Ca^{2+} into Co^{2+}-positive cortical neurones, when
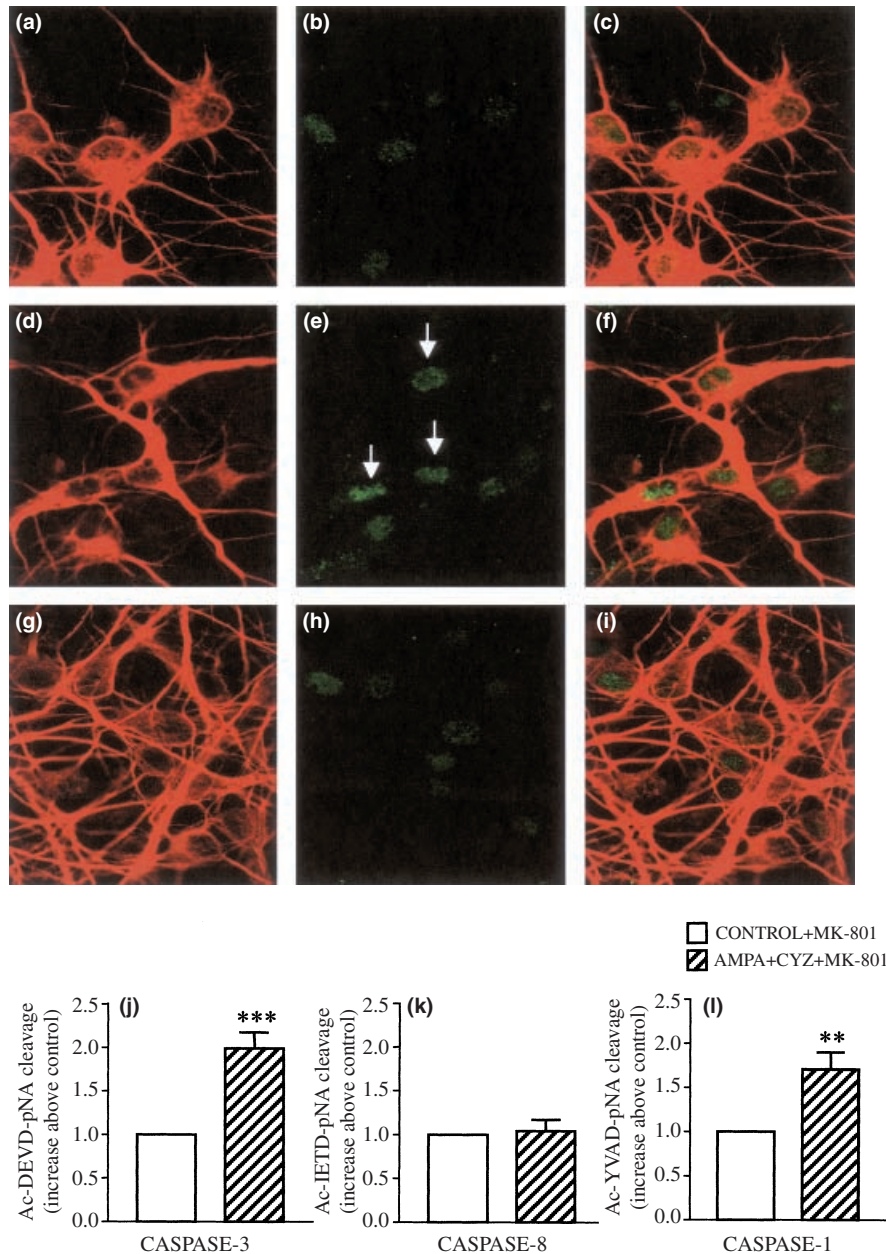
Fig. 6 Analysis of caspase activation upon exposure to AMPA under non-desensitizing conditions. (a–i) Caspase-3 activation was determined by immunocytochemistry (a,d,g: anti-MAP-2 in red; b,e,h: anti-active caspase-3 in green; c,f,i: merged images) in control hippocampal cells (a–c) and upon exposure to 100 μM AMPA + 30 μM CYZ + 10 μM MK-801 in the absence (d–f) or in the presence (g–i) of 20 μM NBQX. Active caspase-3 is visualized more in the nucleus of treated cells, but this is prevented by NBQX. (j–l) Activation of caspases upon selective activation of non-desensitizing AMPA receptors, in the presence of CYZ and MK-801. The activation of caspases-3, -8 or -1 was determined in the presence of the colorimetric substrates Ac-DEVD-pNA, Ac-IETD-pNA and Ac-YVAD-pNA respectively. Values are mean ± SEM of three to four experiments. **$p < 0.01$, ***$p < 0.001$ versus control (unpaired student's *t*-test).

compared with NMDA stimulation, which was shown to be critical for determining neuronal injury (Lu *et al.* 1996). Interestingly, the composition of AMPA receptors was changed upon global ischaemia in the hippocampus, namely through a reduction in GluR2 (but not GluR1) subunits in CA1 neurones, a hippocampal area selectively affected by isch-aemia, although not in CA3 pyramidal cells or in granule cells of the dentate gyrus (Opitz *et al.* 2000). Moreover, the expression of GluR2-lacking Ca^{2+}-permeable AMPA receptors was closely related to an increase in Ca^{2+} influx mediated by activation of AMPA receptors in CA1 neurones exhibiting action potentials (Gorter *et al.* 1997), which are committed to

(a)

(b)
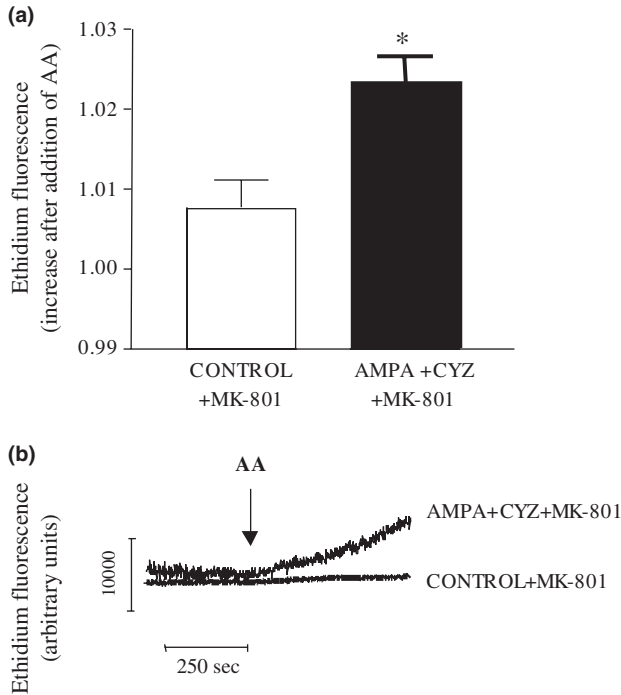
Fig. 7 Superoxide generation upon incubation with AMPA in the presence of CYZ and MK-801 in a population of hippocampal neurones. (a) Superoxide generation was determined with hydroethidine (5 μM) in control and 100 μM AMPA + 30 μM CYZ + 10 μM MK-801-treated hippocampal neurones, after exposure to AA (5 μM), as shown in fluorescence traces of representative experiments (b). Values are mean ± SEM of three experiments. *$p < 0.05$ versus control (unpaired student's *t*-test).

die after ischaemia. It should be noted that, although the hippocampal cultures used in this study contain about 15–20% Ca^{2+}-permeable AMPA receptors (in the absence of the Glu2 subunit), these cultures have been reported to show an increase in the expression of GluR2-containing AMPA receptors during development, after about 7–14 days in culture, related to a lower Ca^{2+} permeability (Pickard *et al.* 2000).

Apart from inducing fast necrotic neuronal death, the increase in intracellular Ca^{2+} concentration induced by stimulation with kainate, shown to interfere with the

mitochondrial membrane potential (Rego *et al.* 2001), may well induce apoptotic neuronal death through the release of cytochrome *c* and activation of the caspase cascade, as shown in this work. Thus, apoptosis probably occurs through AMPA receptor-dependent release of mitochondrial cytochrome *c* (Fig. 5), a first potential trigger for activation of the caspase cascade, as observed by the activation of the executioner caspase-3 (Fig. 6). The observation of active caspase-3 localized in the nucleus may be accounted for by caspase-3-dependent regulation of caspase-activated Dnase/DNA fragmentation factor 40 activity, a nuclease known to degrade chromosomal DNA into nucleosomal fragments in the nucleus (Lechardeur *et al.* 2000). Furthermore, because activation of caspase-3 may also result from the activation of caspase-1 or caspase-8, the results point to caspase-1 (Fig. 6l) as a second potential trigger of caspase-3 activation. Although caspase-1 has been be associated with inflammatory processes (e.g. Furlan *et al.* 1999), which may be related to increased proliferation of microglia, staurosporine-induced apoptosis of cultured rat hippocampal neurones was previously demonstrated to involve the activation of caspase-1 as an upstream initiator of the apoptotic cascade (Krohn *et al.* 1998). In addition, we have shown that the extrinsic apoptotic pathway, mediated by the death receptors and responsible for activating caspase-8, which can activate other caspases or cross-talk with the intrinsic mitochondrial pathway through the cleavage of Bid (Putcha *et al.* 2002), is not activated during selective AMPA receptor stimulation in hippocampal neurones. The importance of apoptotic cell death upon AMPA receptor-mediated excitotoxicity was previously demonstrated by the observation that overexpression of Bcl-2 in cultured cortical neurones protected against apoptosis induced by AMPA receptor activation, but not against necrosis induced by hydrogen peroxide (Cheung *et al.* 2000). Furthermore, apoptotic nuclear features of cell death induced by activation of AMPA receptors were previously observed in cerebellar granule cells (Cebers *et al.* 1997) or in cortical neurones (Larm *et al.* 1997).

Activation of non-desensitizing AMPA receptors was also shown to predispose the hippocampal neurones to a moderate increase in mitochondrial superoxide, as deter-

Table 2 Formation of intracellular peroxides in hippocampal neurones exposed to AMPA, under non-desensitizing conditions

	Control	Control + H_2O_2	A/C	A/C + H_2O_2
DCF fluorescence	1.044 ± 0.010	1.362 ± 0.084*	1.031 ± 0.008	1.185 ± 0.044*
Rhodamine-123 fluorescence	1.319 ± 0.025	3.760 ± 0.141*	1.330 ± 0.031	3.717 ± 0.269*

Values are mean ± SEM increase above the initial fluorescence values (three to five experiments). Hippocampal neurones were incubated with 100 μM AMPA plus 30 μM CYZ (A/C), in the presence of MK-801 (10 μM), for 15 min. Control cells were incubated with MK-801 only. The cells were further incubated in Neurobasal medium for 24 h, in the absence of the drugs. The formation of intracellular peroxides was determined with the fluorescent probes DCFH₂-DA (DCF fluorescence) or DHR-123 (rhodamine-123 fluorescence). Where indicated, intracellular peroxide formation was determined after incubation with 10 mM H_2O_2 , which was used to evaluate the oxidation status of the cells. *$p < 0.001$ versus neurones incubated in the absence of H_2O_2 (one-way ANOVA followed by Tukey *post-hoc* test).
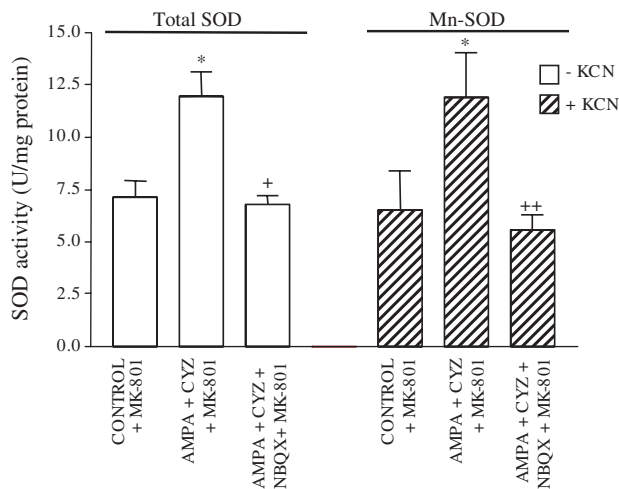
Fig. 8 SOD activity upon selective stimulation of AMPA receptors under non-desensitizing conditions. The neurones were incubated with 100 μM AMPA in the presence of 30 μM CYZ + 10 μM MK-801, in the absence or presence of NBQX (20 μM). In order to distinguish Mn-SOD from Cu/Zn-SOD, the samples were incubated with KCN, an inhibitor of Cu/Zn-SOD. Values are mean ± SEM of three to four experiments. $*p < 0.05$ versus respective control; $+p < 0.05$, $++p < 0.01$ versus AMPA + CYZ + MK-801-treated cells (one-way ANOVA followed by Tukey *post-hoc* test).

mined with hydroethidine, which is oxidized to ethidium, redistributing across the mitochondrial membrane according to the transmembrane potential (Rottenberg 1984). In this study, the increase in ethidium fluorescence in a population of hippocampal neurones was observed only after addition of AA, similar to data obtained in single cerebellar granule cells (Budd *et al.* 1997), whereas further addition of carbonyl cyanide 4-trifluoro methoxy-phenyl-hydrazone (FCCP), a mitochondrial uncoupler, did not change significantly the overall ethidium fluorescence (not shown). Production of superoxide has been associated with increased NMDA or kainate excitotoxicity, namely through the inactivation of aconitase, a key enzyme in the citric acid cycle, which can lead to the disruption of mitochondrial energy production and cell death (Patel *et al.* 1996). Moreover, activation of AMPA receptors was previously shown to enhance the production of mitochondrial superoxide anions (Bindokas *et al.* 1996; Carriedo *et al.* 2000; A.C. Rego and D.G. Nicholls, unpublished results). A small molecule such as superoxide, generated by the xanthine–xanthine oxidase system, has also been associated with the potentiation of synaptic transmission in the CA1 area of the hippocampus, requiring the activation of protein kinase C (Knapp and Klann 2002), a process that may be envisaged as a natural role for superoxide. Furthermore, an enhancement in superoxide formation may also form the basis for cytochrome *c* release and subsequent hippocampal neurodegeneration induced by AMPA

plus CYZ, as reported by Atlante *et al.* (2000) in cerebellar granule neurones undergoing glutamate-induced excitotoxicity. Otherwise, superoxide generation may occur secondary to cytochrome *c* release, as observed during NMDA neurotoxicity (Luetjens *et al.* 2000).

In this study, the increase in Mn-SOD activity can be regarded as a neuroprotective strategy to overcome mitochondrial superoxide production. Mn-SOD, localized predominantly in the neurones throughout the brain (Lindenau *et al.* 2000), was previously shown to mediate protection against NMDA- and nitric oxide-induced neurotoxicity (Gonzalez-Zulueta *et al.* 1998). Overexpression of Mn-SOD was also shown to attenuate neuronal death in a model of amyotrophic lateral sclerosis, a progressive neurodegenerative disease associated with a mutation in the gene encoding the cytosolic Cu/Zn-SOD (SOD1) (Flanagan *et al.* 2002). Moreover, an increased expression of Cu/Zn- SOD was shown to reduce oxidative stress and attenuate apoptotic cell death by decreasing mitochondrial cytochrome *c* and second mitochondrial activation of caspases (Smac) release, and the activation of caspases (Sugawara *et al.* 2002), in agreement with previous studies that showed that SOD delayed neuronal apoptosis induced by nerve growth factor deprivation (Greenlund *et al.* 1995). Although increased generation of H_2O_2 by Mn-SOD might result in oxidative stress and neurotoxicity produced by the highly reactive hydroxyl radical, through the Fenton reaction, no changes in intracellular peroxide were observed, suggesting that severe oxidative stress may not determine AMPA-mediated neuronal injury. In accordance with this, we have observed that treatment of hippocampal neurones with the antioxidants vitamin E or idebenone did not prevent AMPA/CYZ-mediated cell death (data not shown).

In summary, the present study may help us to understand the susceptibility of hippocampal neurones to excitotoxicity mediated by AMPA receptor stimulation, by defining the downstream pathways of cell death occurring during neurodegeneration caused by epileptic seizures, ischaemia or Alzheimer's disease.

Acknowledgements

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