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Neuroprotection by metabotropic glutamate receptor agonists: LY354740, LY379268 and LY389795

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Abstract

In rat cortical neuronal cultures, metabotropic glutamate (mGlu) receptor agonists: LY354740 (+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate); LY379268 (−)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate, and LY389795 (−)-2-thia-4-aminobicyclo[3.1.0]-hexane-4,6-dicarboxylate, were neuroprotective against toxicity induced by *N*-methyl-D-aspartic acid (NMDA), kainic acid and staurosporine as measured by release of lactate dehydrogenase (LDH) activity into culture supernatants and DNA fragmentation by oligonucleosome formation. The potencies of the agonists were at least 100 times greater in reducing nucleosome formation than LDH release indicating a differential effect on neurons dying by apoptosis than by necrosis. In vivo studies showed that LY354740 was able to mediate a partial protection against apoptosis in CA1 hippocampal cells under ischaemic conditions where substantial CA1 cell loss occurred. The effects of the agonists in vitro were: (a) reversed by mGlu receptor antagonist LY341495, (b) enhanced by the presence of glial cells, (c) abrogated by RNA and protein synthesis inhibitors, and (d) unaltered by inhibition of endogenous adenosine activity. These results suggest that group II mGlu receptor agonists may represent a novel therapeutic strategy for the treatment of neurodegenerative diseases.

Keywords: Group II mGlu receptor agonist; Glutamate; Excitotoxicity

1. Introduction

Glutamate is established as the major neurotransmitter in the mammalian brain which mediates fast synaptic transmission and induces neuronal plasticity (for review, see Conn and Pin, 1997). Glutamate receptors are classified into two main types ionotropic and metabotropic. The ionotropic glutamate receptors (iGlu) are homomeric or heteromeric cation-specific ligand-gated ion channels and are further divided into three subgroups based on the potency of the agonists that cause their activation: *N*-methyl-D-aspartic acid (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainic acid. Metabotropic glutamate receptors (mGlu) are G-protein-coupled receptors which comprise eight different subtypes and are also divided into three subgroups defined on the basis of their amino acid sequence homology, pharmacology and the signal transduction pathway to which they are coupled. Group I ($mGlu_1$ and $mGlu_5$) are coupled to the phosphoinositide hydrolysis/calcium $[Ca^{2+}]_i$ mobilisation signal transduction pathway. Group II ($mGlu_2$ and $mGlu_3$) and group III ($mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$) are negatively coupled to cyclic AMP formation.

Excessive activation of glutamate receptors leading to excitoxicity is of primary concern because of its involvement in stroke, trauma, neurodegeneration and epilepsy. Excitotoxicity is thought to occur as a result of a massive increase in glutamate release and the subsequent rise in intracellular Ca^{2+} to pathological concentrations mediated through action on NMDA and AMPA receptor ion channels as well as mGlus which are coupled to phospholipase C activation and release of Ca^{2+} from intracellular stores

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(Choi and Hartley, 1993). Ca^{2+} also enters neurones through voltage-dependent Ca^{2+} channels (VDCCs) which open in response to cellular depolarisation (Siesjö, 1992). As a result, intracellular Ca^{2+} concentrations attain 'overload' concentrations which lead to activation of Ca^{2+}-dependent degradative enzymes and disruption of mitochondrial function (Boxer and Bigge, 1997). Several reports have shown that activation of group II or group III metabotropic receptors are neuroprotective in a number of in vitro models of neuronal degeneration. These include cell death induced by: NMDA and kainate (Bruno et al., 1994; Altemus et al., 1995; Bruno et al., 1995; Orlando et al., 1995; Turetsky et al., 1995; Buisson et al., 1996; Battaglia et al., 1998), β-amyloid peptide (Copani et al., 1995), hypoxia/glucose deprivation (Buisson and Choi, 1995), nitric oxide (Maiese et al., 1994) and staurosporine (Buisson et al., 1994). In vivo studies with aminocyclopentane-dicarboxylic acid (ACPD), a mixed agonist of all mGluRs, has been shown to produce contradictory effects. On the one hand, local infusion of 1*S*,3*R* ACPD into rat brain causes neuronal degeneration (McDonald and Schoepp, 1992; McDonald et al., 1993), whereas others have shown that ACPD is neuroprotective in a mouse model of focal ischaemia (Chiamulera et al., 1992) and can also attenuate acute traumatic spinal cord injury (Theriault, 1997). Studies with the group II agonist (2*S*,1*R*,2*R*,3*R*)-2-(2,3-dicarboxycyclopropyl)-glycine (DCG-IV) have shown that group II mGlu activation reduced limbic motor seizures and neuronal degeneration induced by kainate injection in rats (Shinozaki et al., 1994). In a recent study, LY354740, a potent and selective agonist of $mGlu_2$ and $mGlu_3$, reduced damage to CA1 hippocampal neurons in a gerbil model of global ischaemia (Bond et al., 1998). Although it is agreed that group II mGlu receptors are neuroprotective, the mechanism(s) and the relative contribution of the receptor subtypes: $mGlu_2$ and $mGlu_3$ to mediation of neuroprotection are not clear. It is known that $mGlu_2$ receptors are located presynaptically and can negatively modulate glutamate release (Battaglia et al., 1997) and that this may be a possible means of reducing excitotoxicity (Schoepp and Conn, 1993). In addition, $mGlu_3$ are located on glial cells and their activation may be responsible for induction of the release of a neuroprotective factor (Bruno et al., 1997, 1998).

In this study, highly potent and selective agonists of group II mGlu receptors: LY354740 (Monn et al., 1997), and LY379268 and LY389795 (Monn et al., 1999) have been used to examine the neuroprotective potential of group II mGlu receptors against NMDA and non-NMDA receptor-induced excitotoxicity in rat cortical neuronal cell cultures. Furthermore, we have also evaluated the effects of LY354740 against ischaemia-induced hippocampal cell death in an in vivo model of cerebral ischaemia. Our results show that activation of $mGlu_{2/3}$ subtypes is effective at attenuating neuronal cell death brought about by apoptotic as well as necrotic pathways.

2. Materials and methods

2.1. Materials

NMDA, kainate, AMPA, cyclothiazide, 1,3-dipropyl-8-phenylxanthine (DPPX) and 4-(2-[7-amino-2-(furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385) were obtained from Tocris (UK). (+)-5-Methyl-10,11-dihydro-5*H*-dibenzo-[*a*,*d*]-cyclohepten-5,10-imine hydrogen maleate (MK801) was purchased from RBI (Natick, MA, USA). Cytosine arabinoside, actinomycin D and cycloheximide were obtained from Sigma (UK). Cell culture media and supplements were purchased from Life Technologies (UK). Compounds: LY354740 (+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate; LY379268 (−)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate; and LY389795 (−)-2-thia-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate, were synthesised at Eli Lilly and dissolved in equimolar NaOH to facilitate solution.

2.2. Neuronal cell cultures

Cortical cell cultures were prepared from foetal rat brains at 18 days of gestation. Dissociated cortical cells were plated in 15 mm 24-well vessels (3×10^5 cells/well) using a plating medium of Neurobasal medium containing 10% heat-inactivated foetal calf serum, and glutamine (1 mM). Cultures were kept at 37°C in a humidified 5% CO_2 atmosphere. After 2 days in vitro, nonneuronal cell division was halted by exposure to cytosine arabinoside (5 μM) in serum-free medium containing B27 supplement (Life Technologies) and resulted in cultures containing 15–20% glial cells. In some experiments, mixed neuronal: glial cells were obtained by 14 days of continuous culture of unselected cells prepared from 1-day neonate cortices in Dulbecco's Modified Essential Medium (DMEM) containing 10% foetal calf serum (Rose et al., 1993). Dissociated cortical cells (3×10^5 cells/well) were then added to glial cell monolayers and treated with cytosine arabinoside from day 0 of culture. Pure neuronal cell cultures containing $<5\%$ glial cells were obtained by addition of cytosine arabinoside (1 μM) on day 0 of cortical cell isolation. During the culture period, half the culture medium was replaced at 3–5 day intervals and the cultures were maintained for 10–14 days prior to use for experiments.

2.3. Excitotoxicity

''Fast'' NMDA excitotoxicity (Bruno et al., 1994) was induced by incubating the cultures at 37°C in a magnesium-free Hanks-balanced salt solution containing $CaCl_2$ (2.0 mM), HEPES (10 mM) and glycine (10 μM) with NMDA (300 μM) for 10–15 min at 37°C. The cultures were then returned to serum-free DMEM culture medium supplemented with HEPES (10 mM) and glycine (10 μM) and further incubated for 20–24 h before evaluation of

NMDA toxicity. ''Slow'' excitotoxicity (Bruno et al., 1994) was induced by incubation of cultures with either NMDA (30 μM), kainate (100 μM) or AMPA (100 μM) plus cyclothiazide (10 μM) in DMEM medium without serum for 24 h. MK801 was added to both kainate and AMPA-treated cultures to reduce secondary activation of NMDA receptors.

2.4. Compound treatment

Group II agonists were added to cultures for 15 min prior to addition of NMDA. In cultures subjected to an acute NMDA exposure, the compounds were removed at the same time as NMDA. Under conditions of slow excitotoxicity, agonists were coincubated with the excitotoxin during the 24-h incubation period.

2.5. Assessment of neuronal cell injury

Neuronal cell viability was evaluated by trypan blue dye staining of dead cells. Three fields of neurons were examined per culture well counting a minimum of 100 cells per field. In addition, supernatants from cultures were collected at the end of the 24-h incubation period and the lactate dehydrogenase (LDH) activity which was released from damaged or dead cells was measured by spectrophotometric assay using a cytotoxicity detection kit (Boehringer-Mannheim, UK). EC_{50} values were determined by nonlinear regression analysis using Minitab software. DNA fragmentation was quantified by using an immunoassay kit for measurement of mononucleosome and oligonucleosome formation (Calbiochem/Novabiochem).

2.6. Animals and surgery

Male Mongolian gerbils (Bantin and Kingman, Hull, UK), at least 3 months old and weighing in excess of 60 g, were used. The animals were maintained in standard lighting conditions and food and water were available ad libitum. The animals were anaesthetised with a 5% halothane/oxygen mixture and maintained using 2% halothane delivered with oxygen at 1 l/min via a face mask throughout the operation. Through a midline cervical incision, both common carotid arteries were exposed and freed from surrounding connective tissue. In animals to be rendered ischaemic, both common carotid arteries were clamped for either 3, 4 or 5 min. At the end of the occlusion period, blood flow was reestablished. In sham-operated animals, the arteries were exposed but not occluded. The wound was then sutured and the animals allowed to recover. Throughout surgery, body temperature was maintained at 37°C using a ''K-TEMP'' temperature controller/heating pad (International Market Supply, Cheshire, UK). After surgery, the animals were placed in a four compartmental thermacage (Beta Medical and Scientific, UK) which maintained the environmental temperature at 28°C, and rectal temperatures were measured for a 6-h period after occlusion. LY354740 was administered at

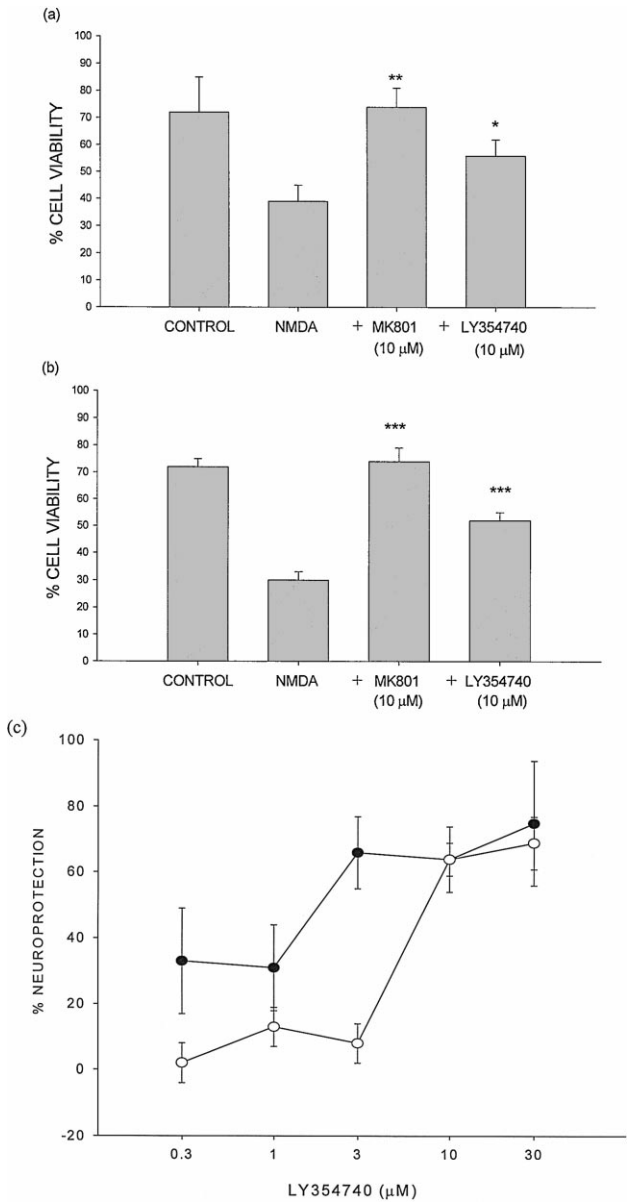

Fig. 1. Pretreatment of rat cortical neuronal cell cultures with LY354740 attenuates NMDA-induced excitotoxicity. Embryonic rat cortical cells were cultured for 12 days as described in Section 2. LY354740 was added to cultures and incubated for 15 min at 37°C. Excitoxicity was initiated by the addition of NMDA at (a) 300 μM for 10 min or (b) 30 μM for 24 h. Results represent the percentage cell viability (mean ± S.E.M. of three separate experiments using duplicate determinations) measured by counting live and dead cells assessed by trypan blue dye exclusion. Results represent the percentage neuroprotection[#] measured by LDH release into the culture supernatants (mean ± S.E.M. of five to seven separate experiments using triplicate culture replication). Open circles represent cultures exposed to NMDA at 300 μM for 10 min; closed circles represent cultures exposed continuously to 30 μM NMDA for 24 h. Statistical analysis was undertaken by two-tailed Student's *t*-test and compare differences between compound-treated cultures and NMDA controls: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. [#]% Neuroprotection = 100 × [1 − (OD test − OD control)/(OD NMDA-treated − OD control).

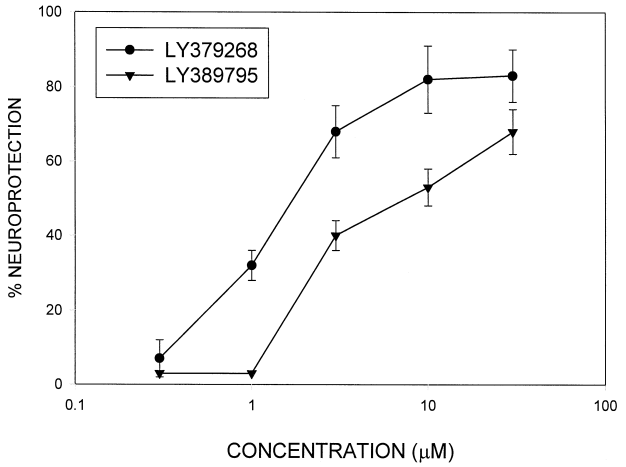

Fig. 2. Novel agonists LY379268 and LY389795 attenuate NMDA toxicity in vitro. Embryonic rat cortical cells were cultured for 12 days as described in Section 2. Agonists were added to cultures and incubated for 15 min at 37°C. Excitoxicity was initiated by the addition of NMDA at 300 μM for 10 min and LDH release was assessed 24 h later. Results represent the percentage neuroprotection calculated as described in the legend to Fig. 1 (mean ± S.E.M. of three experiments using triplicate culture replication).

50 mg/kg, i.p. 30-min post-occlusion, with a second dose of 50 mg/kg, i.p. administered 6 h following occlusion. There were eight animals in each group and sham-operated animals underwent the full surgical procedure, except for the arterial occlusion. Control animals were occluded for 3, 4 or 5 min, but received vehicle only. In separate experiments, control animals underwent bilateral carotid artery occlusion for periods of 2, 3, 4 and 5 min in order to determine the degree of CA1 hippocampal damage resulting from each period of occlusion.

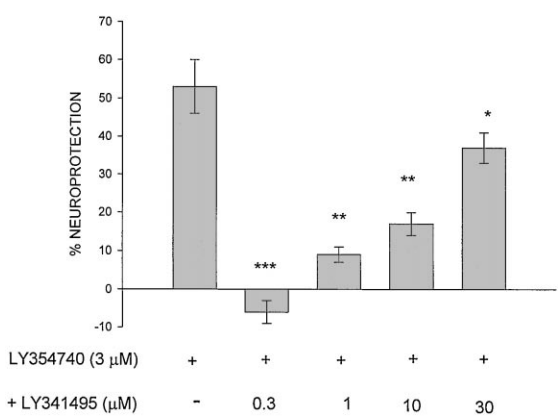

Fig. 3. Antagonist LY341495 reversed the neuroprotective effects of LY354740 at concentrations below 10 μM. Embryonic rat cortical cells were cultured for 12 days as described in Section 2. LY341495 was added to cultures 15 min prior to the addition of LY354740 followed by a further 15-min incubation at 37°C. Excitoxicity was initiated by the addition of NMDA at 300 μM for 10 min and LDH release was assessed 24 h later. Results represent the percentage neuroprotection (mean ± range of two separate experiments) calculated as described in the legend to Fig. 1. Statistical analysis was undertaken by two-tailed Student's *t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ and compare differences in neuroprotection in antagonist-treated vs. control cultures.

2.7. General histology

Five days after surgery, the animals were perfused transcardially with 30 ml of 0.9% saline followed by 100 ml of 10% buffered formalin solution. The brains were removed and placed in 10% formalin for 3 days, processed and embedded in paraffin wax. Coronal sections (5 μm) were taken 1.5, 1.7 and 1.9 mm caudal to bregma using a microtome (Leitz 1400 sledge microtome). The slices were stained with haematoxylin and eosin and the neuronal density in the CA1 subfield of the hippocampus was measured using a microscope with grid lines (0.05 mm × 0.05 mm). The neuronal density is expressed as number of viable cells per millimeter CA1 hippocampus. Statistical analysis of histological data was assessed using a two-tailed unpaired Student's *t*-test, with $P < 0.05$ being considered statistically significant.

2.8. TdT fragment end labelling of DNA

Apoptotic endonucleases affect cellular DNA by producing classical DNA laddering and also generate free 3′-OH groups at the ends of these DNA fragments. Adja-

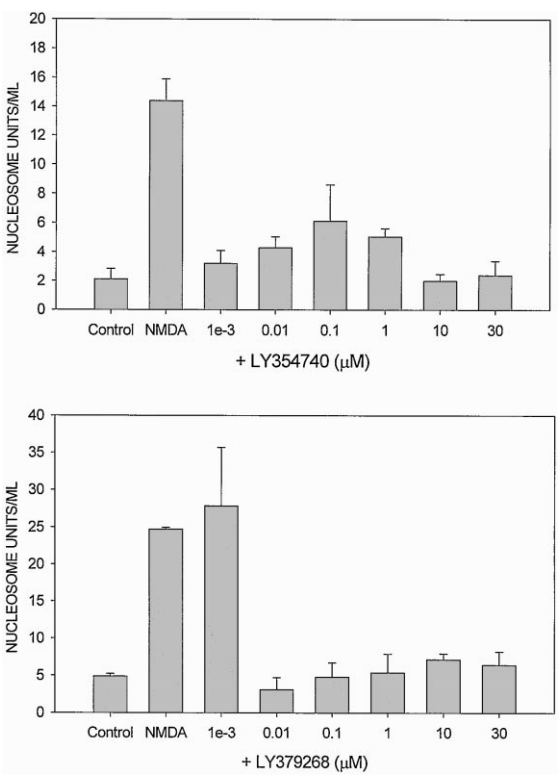

Fig. 4. Group II agonists are effective at preventing apoptosis in NMDA-treated cultures. Embryonic rat cortical cells were cultured for 12 days as described in Section 2. Agonists were added to cultures and incubated for 15 min at 37°C. Excitoxicity was initiated by the addition of NMDA at 30 μM followed by 24-h incubation at 37°C. Apoptosis was measured by quantifying DNA fragmentation using a nucleosome ELISA method. Results show the mean ± S.D. using quadruplicate culture replication.

cent hippocampal sections were end-labelled using FragEL DNA framentation kits (Calbiochem-Novabiochem, UK). Briefly, sections were deparaffinised with washes of xylene before rehydration using decreasing concentrations of industrial methylated spirits (IMS). The sections were then permeabilized by incubating with 20 μg/ml proteinase K and endogenous peroxidases inactivated with 0.3% H_2O_2. Following a 10–20 min of incubation with TdT equilibration buffer, the labelling reaction was performed. TdT labelling reaction mixture was prepared and applied to each section and incubated for 1.5 h at 37°C. After termination of the labelling reaction, the labelled DNA was detected by incubating with a streptavidin horse radish peroxidase conjugate. The reaction product was visualised using diaminobenzine and counterstained with methyl green. Sections were dehydrated and prepared for microscopic examination.

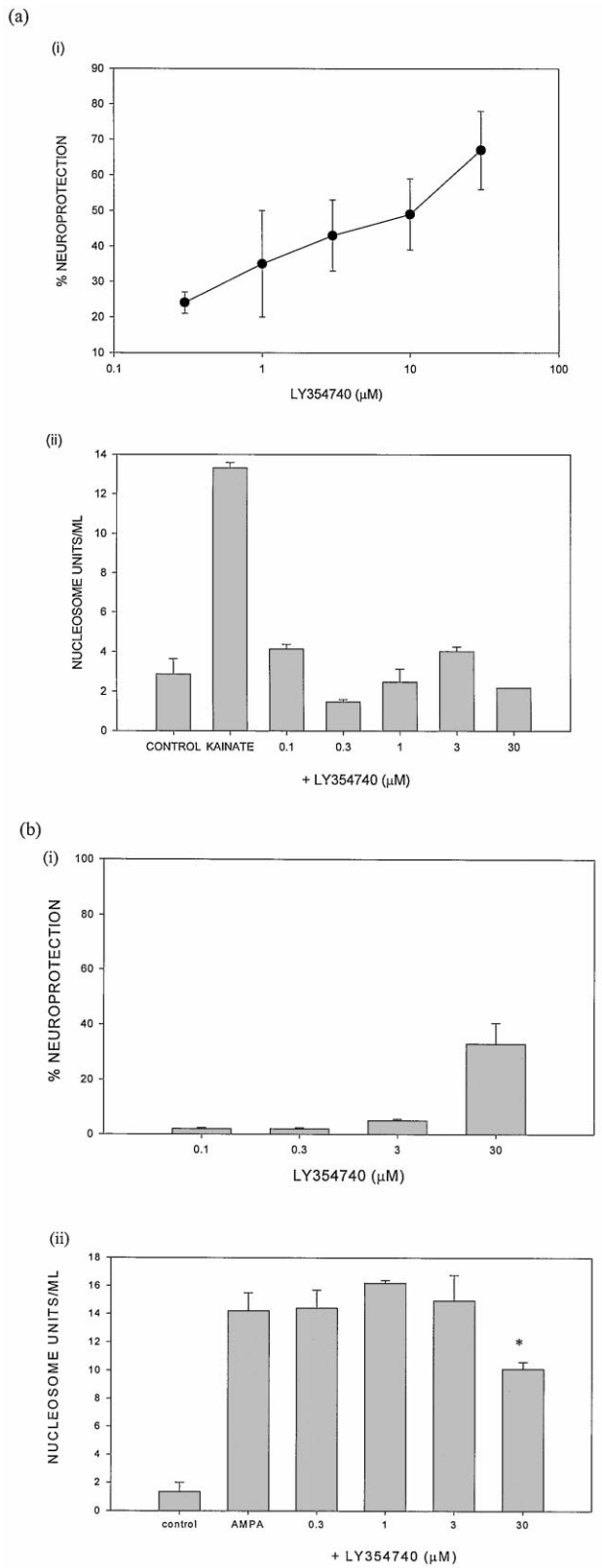

Table 1
Novel group II mGlu receptor agonists protect against kainic acid-induced excitotoxicity

Compound (1 μM)	Percentage neuroprotection against kainic acid treatment
LY379268	45 ± 9^b
LY389795	39 ± 6^a
LY354740	47 ± 4^b

Embryonic cortical neuronal cells (DIV 12) were preincubated with agonists for 15 min at 37°C. Kainic acid (100 μM) was added in the presence of MK801 (10 μM) and further incubated for 24 h. LDH release into the supernatants of the cultures was measured and the percentage neuroprotection versus control cultures was calculated. Results show mean ± S.E.M. of three experiments using triplicate culture replication. $^aP < 0.01$, $^bP < 0.001$ compared to kainic acid alone control cultures.

3. Results

3.1. LY354740, LY379268 and LY389795 protect against NMDA receptor-induced LDH release in neuronal cultures

Initial experiments with LY354740 shown in Fig. 1a and b indicate that the compound was able to preserve the viability of cortical neuronal cells under conditions of excitotoxicity induced by either an acute exposure of cells to a high concentration of NMDA (fast toxicity) or continuous incubation of cells with a low concentration of NMDA (slow toxicity). Further analysis of the neuropro-

Fig. 5. LY354740 protects neurons from kainate but not AMPA-induced excitotoxicity. Embryonic rat cortical cells were cultured for 12 days as described in Section 2. Agonists were added to cultures and incubated for 15 min at 37°C. Excitotoxicity was initiated by the addition of either (a) kainate (100 μM) or (b) AMPA (100 μM) plus cyclothiazide (10 μM) followed by a 24-h incubation in the presence of MK801 (10 μM). Results represent (i) the percentage neuroprotection calculated as described in the legend to Fig. 1 (mean ± S.E.M. of three experiments using triplicate culture replication) and (ii) apoptosis measured by nucleosome formation (mean ± S.D. of quadruplicate culture replication). Statistical analysis was undertaken by two-tailed Student's *t*-test and compare differences between compound-treated cultures and AMPA controls; $^*P < 0.05$.

Table 2
Novel group II agonists protect against staurosporine-induced LDH Release

Compound (1 μM)	Percentage neuroprotection against staurosporine treatment
LY379268	60 ± 4[a]
LY389795	62 ± 5[a]
LY354740	69 ± 9[a]

Embryonic cortical neuronal cells (DIV 12) were preincubated with agonist for 15 min at 37°C. Staurosporine (1 μM) was added and cells were incubated for 24 h. LDH release into the supernatants of the cultures was measured. Results show mean ± S.E.M. of three to four experiments using quadruplicate culture replication.
[a] $P < 0.001$ compared to staurosporine-treated cultures.

tective effects of LY354740 demonstrated that LY354740 produced concentration-dependent neuroprotection against both fast and slow NMDA-induced toxicity in neuronal-enriched rat cortical neurons measured by LDH activity released into the medium (Fig. 1c). LY354740 was moderately less potent in preventing excitotoxicity induced by a chronic exposure to NMDA than for excitotoxicity caused by an acute exposure to a high concentration of NMDA. The EC_{50} value of LY354740 for protection against fast toxicity ranged from 0.9 to 1.3 μM (range of seven experiments) and differed from that obtained for slowly triggered neuronal degeneration with an EC_{50} range of 7–8.2 μM (range of five experiments; $P < 0.05$). The heterobicyclic amino acids: LY379268 and LY389795, produced a similar profile of concentration-dependent neuroprotective effects to LY354740 (Fig. 2). Using the fast NMDA toxicity model, the EC_{50} values of LY379268 and LY389795 for neuroprotection ranged from 0.9 to 2.8 μM and from 5 to 10 μM, respectively (ranges of three experiments).

Addition of the group II mGlu receptor antagonist LY341495 (Kingston et al., 1998) caused a reversal of the neuroprotective actions of LY354740 (Fig. 3). The concentration-dependent effects of LY341495 follow a bimodal course such that at low concentrations LY341495 reverses neuroprotection induced by LY354740 but that at higher concentrations (≥ 10 μM), the compound shows a trend towards neuroprotection most likely due to antagonism of group I mGlu receptors (Kingston et al., 1998). Taken together these results support that the neuroprotective effects of LY354740 are mediated through mGlu receptor activation. LY341495 was also able to reverse the effects of LY379268 and LY389795 (results not shown).

3.2. Group II agonists prevent nucleosome formation in NMDA-treated cortical cell cultures

Evidence has accumulated to show that in vitro neuronal cell death occurring by glutamate receptor activation involves apoptotic processes (Boxer and Bigge, 1997; Du et al., 1997). Apoptosis induces a series of changes that include chromatin condensation and cytoplasmic blebbing. Activation of endonucleases results in the excision of nucleosomes comprising 180–200 base pair units of DNA which can be observed qualitatively as a DNA ladder in agarose gels. In this study, quantitative assay of mononucleosomes and oligonucleosomes has been undertaken by ELISA methodology (Huang and Plunkett, 1992). Results show that NMDA-treated cortical cells exhibit an increase in nucleosome formation by sixfold to tenfold over control

Table 3
The effects of LY354740 against ischaemic-induced: (a) cell loss and (b) DNA fragmentation, in the gerbil hippocampus

(a)

Occlusion time	Sterotaxic level (from bregma) [mm]	Sham control	Ischaemic control	Ischaemic + LY354740
Number of viable cells / mm CA1 hippocampus				
3 min BCAO	1.5	214 ± 6	23 ± 14[a]	156 ± 26[b]
	1.7	216 ± 4	24 ± 14[a]	157 ± 25[b]
	1.9	220 ± 6	25 ± 15[a]	155 ± 25[b]
4 min BCAO	1.5	191 ± 5	9 ± 1[a]	21 ± 6[c]
	1.7	191 ± 3	10 ± 1[a]	26 ± 5[c]
	1.9	194 ± 5	11 ± 1.5[a]	29 ± 6[c]
5 min BCAO	1.5	226 ± 2	10 ± 1[a]	12 ± 1
	1.7	226 ± 3	11 ± 1[a]	10 ± 2
	1.9	220 ± 2.4	13 ± 2[a]	13 ± 2

(b)

Number of apoptotic cells / mm CA1 hippocampus				
3 min BCAO	1.7	1 ± 0.5	116 ± 22.7[a]	8 ± 4.8[b]
4 min BCAO	1.7	1 ± 0.4	148 ± 35[a]	95 ± 13[c]
5 min BCAO	1.7	1 ± 0.3	152 ± 24.5[a]	142 ± 13.6

Data is expressed as mean ± S.E.M.: (a) viable cells/mm CA1 ($n = 8–10$ per group) and (b) apoptotic cells/mm CA1 ($n = 5$ per group). Student's *t*-test.
[a] $P < 0.001$ vs. sham control; [b] $P < 0.01$, [c] $P < 0.05$ vs. ischaemic control.

cultures (Fig. 4). Addition of either LY354740 or LY379268 significantly reduced nucleosomal DNA fragmentation at low nanomolar concentrations, i.e., at lower concentrations than required to reduce LDH release (micromolar). Moreover, it was evident that the magnitude of protection observed at nanomolar concentrations was not significantly improved at higher micromolar concentrations.

3.3. Effects of group II agonists on non-NMDA receptor-induced neuronal excitotoxicity

Treatment of rat cortical neurons with LY354740 reduced kainate-induced cell death as measured by LDH release and nucleosomal formation and as seen previously for NMDA toxicity, the concentrations at which LY354740 prevented nucleosome formation were up to a 1000 fold less than that required for reduction of LDH release (Fig. 5a). As seen for LY354740, the other agonists, LY379268 and LY389795 were also effective in reducing kainate toxicity (Table 1). In contrast, LY354740 was not effective in protecting against AMPA/cyclothiazide-induced cell death at concentrations up to 3 μM. However, at the higher concentration of 30 μM, LY354740 did show a trend towards a reduction in both LDH release and nucleosomal formation (Fig. 5b). Similarly, LY379268 and LY389795 did not protect against AMPA-induced cell death up to concentrations of 10 μM (results not shown).

To assess whether or not pretreatment with the group II agonists could attenuate neuronal cell death induced by a nonglutamate receptor-mediated agent, the effects of the compounds on staurosporine-induced neuronal cell death were investigated. Table 2 shows that all three agonists can protect against staurosporine-induced toxicity with similar potencies of effect.

3.4. Neuroprotective effects of LY354740 against cerebral ischaemia in vivo

In control animals, increasing the time of bilateral carotid artery occlusion resulted in a time-dependent increase in CA1 hippocampal damage (Table 3a). A 3-min occlusion resulted in approximately 80% CA1 neuronal cell loss. A further increase in cell death was observed (> 90%), following 4-min occlusion, this being maintained following 5 min of occlusion. LY354740 produced significant neuroprotection (72%, $P < 0.001$) in animals which had undergone 3-min occlusion. The level of neuroprotection fell steeply to 15% ($P < 0.05$) when the occlusion time was increased to 4 min. Following 5 min of occlusion, no significant neuroprotection (6%) was achieved (Table 3a). Examination of adjacent sections for TdT fragment end-labelling of DNA indicated that there was a large increase in the number of apoptotic cells following 3 min of occlusion and this effect was greater with a 4 or 5-min period of occlusion (Table 3b). LY354740 (50 mg/kg, i.p) almost completely blocked the 3-min ischaemia-induced increase in apoptotic cells. The compound also produced a significant reduction (36%) in the number of apoptotic positive cells following a 4-min period of occlusion, but failed to have any effect when the occlusion time was increased to 5 min (Table 3b).

3.5. Mechanism of group II agonist action

Bruno et al. (1997) have reported that the neuroprotective activity of group II mGlu receptor requires new protein synthesis and glial-neuronal interaction. In this study, a comparison of agonist effects in different types of culture was carried out and results showed that in mixed neuronal and glial cell cultures, the activities of all the agonists were significantly enhanced compared to pure

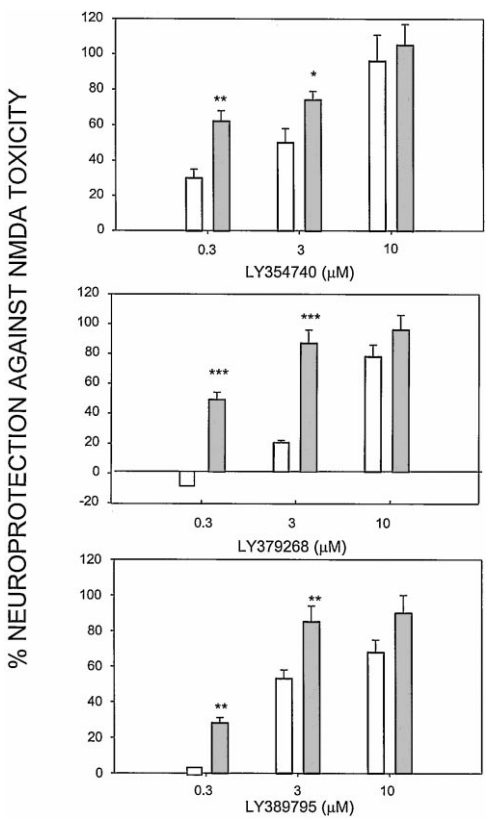

Fig. 6. The neuroprotective effects of group II agonists are augmented in mixed neuronal cell cultures containing glial cells compared to pure neuronal cell cultures. Neuronal cells were prepared from embryonic rat cortices (see Section 2) and cultured in the presence of a low (i.e., < 5%; open bars) or high glial cell concentration (~ 50% glia; grey bars). Agonists were added to the cultures and excitotoxicity was induced by exposure to NMDA (30 μM). LDH release was assessed 24 h later. Results represent the percentage neuroprotection calculated as described in the legend to Fig. 1 (mean ± range of two experiments using triplicate culture replication). Statistical analysis was undertaken by two-tailed Student's *t*-test and compare differences in neuroprotection between neuronal-enriched and glial cell containing cultures $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

neuronal cell cultures with the most marked effects apparent at submicromolar concentrations (Fig. 6). Further investigation of the effects of RNA and protein synthesis inhibitors on the neuroprotective effects of LY354740 was undertaken. Addition of either actinomycin D (0.5 μg/ml) or cycloheximide (1 μg/ml) to LY354740-treated cultures blocked the neuroprotection against NMDA toxicity measured by both LDH release and nucleosome formation (Fig. 7). Group II mGlu receptors have been shown to potentiate responses to endogenous adenosine in hippocampus (Winder and Conn, 1993). Adenosine itself, has been shown to be neuroprotective (Fredholm, 1997; Sweeney, 1997; Logan and Sweeney, 1997) and may therefore be implicated in the mechanism of action of the group II agonists. To investigate this possibility, the effects of LY354740 on NMDA toxicity were measured in the presence of adenosine receptor antagonists: DPPX and ZM 241385. Results in Fig. 8 show that neuroprotection by

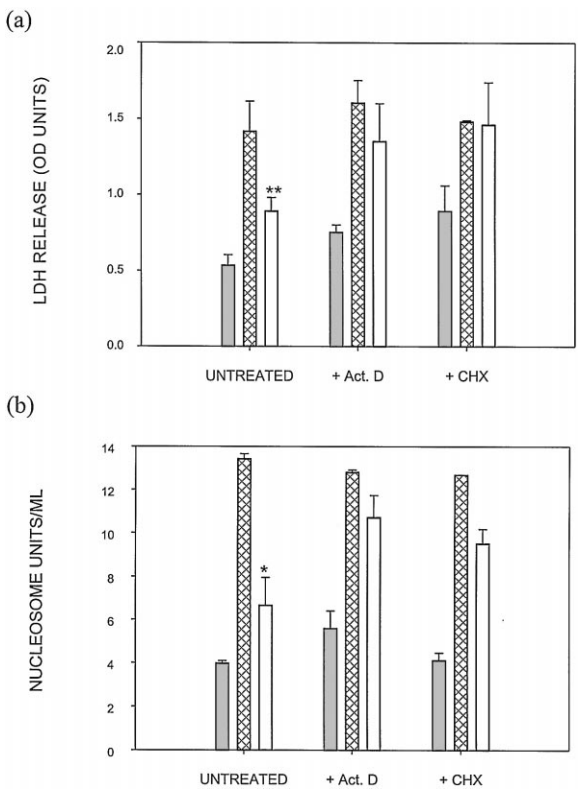

Fig. 7. RNA and protein synthesis inhibitors block the neuroprotective effects of LY354740. Embryonic rat cortical cells were cultured for 12 days as described in Section 2. LY354740 (3 μM) was added to cultures in the presence of either Act. D (0.5 μg/ml) or CHX (1 μg/ml) was incubated for 20 min at 37°C. Excitotoxicity was induced by the addition of NMDA at 30 μM and LDH release was measured 24 h later. Results show (a) LDH release and (b) apoptosis measured by nucleosome formation (mean ± S.E.M. of triplicate culture replication). Untreated control cultures are shown as grey bars; NMDA-treated cultures are shown as hatched bars and LY354740 + NMDA-treated cultures are shown as open bars. Statistical analysis was undertaken by two-tailed Student's *t*-test and compare differences between LY354740-treated cultures and respective NMDA controls; $^{*}P < 0.05$; $^{**}P < 0.01$.

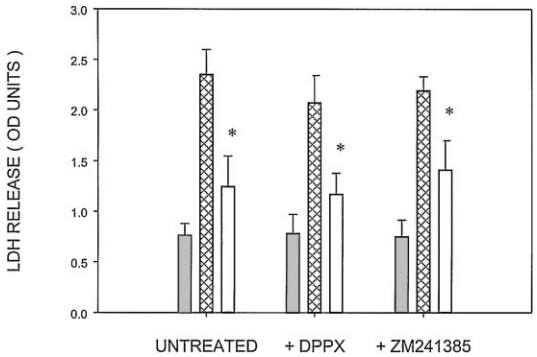

Fig. 8. Embryonic rat cortical cells were cultured for 12 days. DPPX (1 μM; 1,3-dipropyl-8-phenylxanthine) — an A1 receptor antagonist and ZM 241385 (1 μM; 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol) — an A2A adenosine receptor antagonist were added to cultures in the presence or absence of LY354740 (3 μM) and incubated for 15 min at 37°C. Excitotoxicity was initiated by the addition of NMDA at 100 μM followed by 24-h incubation at 37°C. LDH release was then assessed. Results show a representative experiment using quadruplicate culture replication. Untreated control cultures are shown as grey bars; NMDA-treated cultures are shown as hatched bars and LY354740 + NMDA-treated cultures are shown as open bars. Statistical analysis was undertaken by two-tailed Student's *t*-test and compare differences between LY354740-treated cultures and respective NMDA controls; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

LY354740 was not affected by the presence of either antagonist indicating the effects of the compound are independent of adenosine receptor activation.

4. Discussion

A role for group II mGlu receptors in mediating neuroprotection against excitotoxicity has been reported previously. Bruno et al. (1997) and Battaglia et al. (1998) have demonstrated that a number of group II receptor agonists including DCG-IV, (*S*)-4-carboxy-3-hydroxyphenylglycine (4C3HPG), (2*S*,1*S*,2*S*)-2-(carboxycyclopropyl)-glycine (L-CCG-1) and aminopyrrolidine-2*R*,4*R*-dicarboxylic acid attenuate excitotoxic neuronal cell death in mouse cortical neurones grown in mixed cultures. These authors also reported that this protection was blocked by the group II mGlu receptor antagonists: (2*S*)-α-ethylglutamate and (2*S*,1*S*,2*S*,3*R*)-2-(2-carboxy-3-phenylcyclopropyl)glycine (PCCG-IV). In the present study, we show that novel and highly potent agonists of group II mGlu receptors: LY354740, LY379268 and LY389795 also protect against NMDA and kainate-induced toxicity in rat cortical neuronal cell cultures and that the action of LY354740 is reversed by the mGlu receptor antagonist LY341495. LY354740 was slightly less potent in preventing excitotoxicity induced by a chronic exposure to NMDA than for excitotoxicity caused by an acute exposure to a high concentration of NMDA. The observation that the neuroprotective effects of LY354740 showed a discrimination in

terms of potency between fast and slow NMDA-induced excitotoxicity may reflect that the biochemical events mediating rapidly triggered cell death are not equivalent to those involved in the more protracted onset of degeneration and death since excitotoxins may induce multiple pathways leading to cell death (Choi and Hartley, 1993). Interestingly, Buisson et al. (1996) have reported a more extreme contrast between the two experimental paradigms in that DCG-IV has selective neuroprotective effects on fast rather than slow NMDA toxicity and does not protect against kainate toxicity in mouse neuronal cell cultures. These observations warrant further assessment and comparison of the molecular events involved in mediating cell death by slow and fast NMDA toxicity. Further, Turetsky et al. (1995) have shown that in cortical neuronal cultures, mGlu receptors selectively protect Ca^{2+}-permeable AMPA/kainate receptors. Therefore, it is worth considering that some of the discrepancies in the efficacy of mGlu agonist effects may arise because of differences in both ionotropic and mGlu expression in different neuronal culture preparations. In this study, in contrast to protecting against NMDA-induced and kainate-induced toxicity, the agonists did not affect AMPA-mediated excitotoxicity in the presence of cyclothiazide and the NMDA antagonist MK801. These results agree with those of Bruno et al. (1994, 1995). Cyclothiazide has been shown to potentiate the excitotoxic effects of AMPA in neuronal cultures (May and Robison, 1993). In this study, we were unable to induce excitotoxicity in the cortical neuronal cultures by AMPA without the addition of cyclothiazide. It is conceivable that under conditions where receptor desensitisation is prevented, the regulatory neuroprotective effects of group II mGlu receptors become less effective. Further investigation of the selective interaction between group II mGlu receptors and AMPA and kainate receptors in cortical neurons is currently under study.

LY354740 has recently been described as a potent and selective $mGlu_{2/3}$ agonist with EC_{50} values of 5.1 and 24.3 nM, respectively (Schoepp et al., 1997). LY389795 and LY379268 are more potent agonists of group II mGlu receptors than LY354740 (Monn et al., 1999). In comparing the nanomolar potencies of the group II agonists on clonal cell lines expressing $mGlu_2$ (LY379268 $EC_{50} = 2.7$ nM; LY389795 $EC_{50} = 3.9$ nM) and $mGlu_3$ (LY379268 $EC_{50} = 4.6$ nM; LY389795 $EC_{50} = 7.6$ nM) with their neuroprotective activities, major differences in potency of up to 1000-fold were observed with respect to the concentrations of the compounds that caused a reduction in LDH release. Moreover, the discrimination in relative potency between the agonists: LY379268 > LY389795 > LY354740 seen in the cAMP assays using the clonal cell lines was not evident by LDH assay analysis. This difference may be attributable to a number of factors which include the likelihood that in native neuronal cell culture, compound potency may be reduced due to a lower expression of Gi/Go protein-coupled group II mGlu receptors than for heterologous clonal cell lines. A further consideration is that the mechanism of neuroprotective action of the LY compounds may not be directly coupled to inhibition of adenylate cyclase since others have shown that group II mGlu receptors may potentiate cAMP production in brain tissue (Winder and Conn, 1995). Functional consequences of group II mGlu receptor activation may include: presynaptic inhibition of glutamate release by negative modulation of voltage-sensitive Ca^{2+} channels (Chavis et al., 1995); induction of neurotrophic/neuroprotective factors (Bruno et al., 1997), and/or post-synaptic modulation of neuronal susceptibility to excessive glutamate receptor activation via induction of a protective intracellular biochemical pathway. Recent studies have reported that LY354740 completely prevented the veratridine-evoked release of striatal glutamate and aspartate, in vivo, as measured by microdialysis (Battaglia et al., 1997). The excessive increase of extracellular glutamate following ischaemia is thought to play a critical role in the development of neuronal damage (Choi, 1987, 1988; Globus et al., 1988; Butcher et al., 1990). Therefore, presynaptic inhibition of glutamate would be protective in neurodegenerative diseases such as ischaemia. Although, previous in vitro studies have shown that neuroprotection by DCG-IV is not attributable to modulation of glutamate release in cortical cell cultures (Buisson et al., 1996) further experiments are in progress with the LY agonists to investigate this possible mechanism. In this study, the activity of all the agonists were enhanced in mixed neuronal:glia cell cultures compared to neuronal-enriched cultures indicating that glial cells may be augmenting or directly mediating the neuroprotective mechanism(s). These effects may be due to a number of possible reasons which may not be mutually exclusive, e.g., for activation of $mGlu_3$ expressed on glial cells leads to expression of glial cell-derived neuroprotective factors and/or a glial cell-derived factor may cause an enhancement of $mGlu_2$ receptor-mediated signal transduction in neuronal cells which in turn leads to induction of a neuroprotective pathway intrinsic to neurons. In our study, we found that inhibitors of RNA and protein synthesis neutralised the neuroprotective effects of LY354740 against NMDA neurotoxicity indicating that new protein synthesis is important in the mechanism of action of group II mGlu receptor agonists. These results would support the findings of Bruno et al. (1997) which show that group II mGlu receptor-mediated neuroprotection is dependent on the synthesis of a heat-sensitive, transferable neuroprotective factor derived from agonist-treated astrocyte cultures which may be TGF beta (Bruno et al., 1998). Further consideration of glial cell involvement would include the possibility that stimulation of the group II mGlu receptors located on neurons may induce the release of a glial cell metabolite such as adenosine or cAMP itself (Gereau and Conn, 1994). These molecules can also be considered as relevant candidates for counteracting excitotoxicity in vitro and in vivo. However, in this study, adenosine receptor antago-

nists did not affect the neuroprotective actions of LY354740 indicating that group II agonist effects are independent of adenosine-mediated neuroprotection.

Another possible explanation of the discrepancy in neuroprotective potency and relative affinities of the LY compounds for $mGlu_2$ and $mGlu_3$ in clonal cell lines is that their actions are not in fact mediated by group II mGlu receptors. Apart from $mGlu_6$, which is located predominantly in the retina, the only other known receptor with which these molecules interact is $mGlu_8$, which can also couple negatively to adenylate cyclase in heterologous clonal cells (Wu et al., 1998). LY354740, LY379268 and LY389795 all have micromolar potencies on clonally expressed $mGlu_8$ with EC_{50} values of 11.5, 1.7 and 7.3 μM, respectively (Monn et al., 1999). These potencies are comparable to the concentrations of the compounds that were effective in reducing LDH release. It is conceivable, therefore, that the neuroprotection exhibited by the three compounds on LDH release may reflect their activation of $mGlu_8$ in rat cortical neuronal cultures. However, LY354740 and LY379268 were also able to mediate anti-apoptotic effects quantitated by nucleosome ELISA at nanomolar concentrations that were commensurate with their measured receptor affinities in clonal cell lines expressing $mGlu_2$ and $mGlu_3$. These observations suggest that the action of the agonists may be a composite of effects at both group II and group III mGlu receptors such that the neuroprotective consequences of group II mGlu receptor activation differentiates a population of apoptotic cells within the overall population of dying neurons. Further studies with selective antagonists of group II and group III mGlu receptors will facilitate our understanding of these differences in activity. Such a differentiation may also explain the selective effects of the agonists on kainate vs. AMPA-mediated toxicity since apoptosis has been shown to be a major component of the kainate-mediated cell death in cerebellar granule cells (Simonian et al., 1996). Moreover, evidence to support an anti-apoptotic effect is given by the ability of the LY compounds to protect against staurosporine-induced neuronal cell death which is considered to be mediated primarily through activation of apoptotic pathways (Koh et al., 1995). Further, the results from in vivo analysis of the effects of LY354740 on apoptotic vs. necrotic cells within the CA1 pyramidal cells of the ischaemic hippocampus also indicated a differential effect by the group II agonist on apoptotic and necrotic neurons. The compound showed a reduction in the number of apoptotic cells under circumstances of a 4-min carotid artery occlusion where the ischaemic damage had caused $>80\%$ cell loss. Taken together, the in vitro and in vivo results obtained in this study would support that the mechanism of action of the neuroprotection by group II agonists is directed against a key intracellular event that is part of a cascade of biochemical events contributing to apoptotically driven cell death. As a consequence, these findings add to the case for continued investigation of the emerging neuroprotective potential of group II mGlu receptor agonists under circumstances of protracted neurodegeneration where delayed cell death contributes to the neuropathophysiology.

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