Br. J. Pharmacol. (1991), **103**, 1819–1823 © Macmillan Press Ltd, 1991

Neuroprotective actions of GR89696, a highly potent and selective κ-opioid receptor agonist

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1 The effect of a novel, highly potent and selective κ-opioid receptor agonist, GR89696, has been evaluated in two animal models of cerebral ischaemia: transient bilateral carotid artery occlusion in the Mongolian gerbil and permanent, unilateral middle cerebral artery occlusion in the mouse.

2 In the Mongolian gerbil model, administration of GR89696 (3 to 30 $\mu g\,kg^{-1}$, s.c.), immediately before and at 4 h after insult, produced a dose-dependent reduction in the hippocampal CA_1 neuronal cell loss resulting from a 7-min bilateral carotid occlusion. Similar effects were obtained with two other κ-agonists, GR86014 (1 $mg\,kg^{-1}$, s.c.) and GR91272 (1 $mg\,kg^{-1}$, s.c.). The neuroprotective effect of GR89696 was completely blocked by prior administration of the opioid receptor antagonist, naltrexone, at 10 $mg\,kg^{-1}$, s.c. Repeated post-treatment with GR89696 (100 $\mu g\,kg^{-1}$, s.c.) or GR44821 (10 $mg\,kg^{-1}$, s.c.) was also effective in protecting completely the hippocampal CA_1 neurones from ischaemia-induced neurodegeneration.

3 In the permanent, unilateral middle cerebral artery occlusion model in the mouse, repeated administration of GR89696 at 300 $\mu g\,kg^{-1}$, s.c. produced a 50% reduction in cerebrocortical infarct volume. In these experiments GR89696 was dosed 5 min, 4, 8, 12, 16, 20 and 24 h after occlusion on the first day and then three times daily for the next three days. GR89696 (300 $\mu g\,kg^{-1}$) also produced a significant 35% reduction in infarct volume in this model when the initiation of dosing was delayed for 6 h after the insult.

4 The results indicate that the potent κ-opioid receptor agonist, GR89696, is neuroprotective in both global and focal cerebral ischaemia models and suggest that, with this class of compound, there may be a considerable time window for pharmacological intervention.

Keywords: κ-opioid agonists; neuroprotection; cerebral ischaemia; GR89696; naltrexone

Introduction

Opioid receptors have been divided into three subtypes: mu (μ), kappa (κ) and delta (δ) and it is well established that activation of each of these receptors can produce antinociceptive effects in a variety of animal models (Hayes *et al.*, 1987; Dickenson *et al.*, 1988; Rodriguez *et al.*, 1986). Within the last decade a number of compounds with enhanced selectivity for the κ-opioid receptor have been described: U50488H (Vonvoigtlander *et al.*, 1983), U62066E (Peters *et al.*, 1987), PD117302 (Leighton *et al.*, 1987), ICI199441 (Costello *et al.*, 1988) and CI-977 (Hunter *et al.*, 1990). It has been suggested that agonists selective for the κ-opioid receptor, while producing good analgesic activity, will lack many of the side-effects associated with μ-opioid receptor agonists.

In addition to playing a role in antinociception, it has been suggested that activation of the κ-opioid receptor can produce neuroprotective effects in certain animal models of cerebral ischaemia. For instance, Tang (1985) and Hall & Pazara (1988) reported that U50488H and U62066E reduced the hippocampal CA_1 neuronal necrosis resulting from transient (7 or 10 min) bilateral carotid occlusion in the Mongolian gerbil. Further studies showed that U50488H also reduced the cerebral oedema and mortality resulting from a 4 h bilateral carotid occlusion in Fischer 344 rats (Silvia & Tang, 1986; Silvia *et al.*, 1987) and the cerebral oedema, cerebrocortical and striatal neuronal cell loss and the mortality resulting from transient (1 h) or permanent middle cerebral artery occlusion in the cat (Silvia & Tang, 1986; Tang & Silvia, 1986). Similarly, CI-977 has been shown recently to reduce infarct volume in a rat middle cerebral artery occlusion model (Cordon *et al.*, 1990). Thus, clinical use of κ-opioid receptor agonists may provide a novel approach for the treatment of stroke.

Recently, our own laboratory has described the chemistry and pharmacology of a series of highly potent and selective κ-opioid receptor agonists (Hayes *et al.*, 1990; Naylor *et al.*, 1990; Scopes *et al.*, 1990). The aim of the present study was to evaluate the efficacy of these compounds in two animal models of cerebral ischaemia: transient bilateral carotid artery occlusion (BCO) in the Mongolian gerbil (Kirino, 1983) and permanent unilateral middle cerebral artery occlusion in the mouse (Gotti *et al.*, 1989). Some of these data have appeared in abstract form (Birch *et al.*, 1990).

Methods

Transient bilateral carotid artery occlusion in the Mongolian gerbil

Mongolian gerbils, of either sex, weighing 60–100 g were anaesthetized with a mixture of isoflurane (5% induction; 2% maintenance), 70% nitrous oxide and 30% oxygen. A 1–2 cm midline cervical incision was made to expose both carotid arteries lying lateral to the trachea. Each carotid artery was carefully isolated from the vagus nerve and a length of cotton thread was placed loosely around it. Once both carotid arteries had been dissected free from accompanying structures, they were occluded for 7 min by the use of microaneursym clips. At the end of the occlusion period, the clips were removed, the patency of the arteries assured by visual inspection and the incision was closed with two wound clips. Sham-operated animals underwent anaesthesia and surgery but the arteries were not occluded. Seven days later, animals were killed by cervical dislocation and the brains removed and rapidly frozen. Coronal sections (20 μm), containing the dorsal hippocampus (1 to 3 mm posterior to Bregma), were then cut

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on a cryostat at 80 to 120 μm intervals. Sections were placed on a microscope slide, fixed in buffered 10% formalin for 10 min and then stained with haematoxylin. Slides were then coded and the area of staining of the CA_1 hippocampal neuronal cell layer was determined with an image analyser (Quantimet 970). Results are expressed as the mean area (μm^2) of staining ± s.e.mean.

Permanent unilateral middle cerebral artery occlusion in the mouse

Male mice (Glaxo-bred CRH) weighing 18–22 g were anaesthetized with a mixture of isoflurane (5% induction; 2% maintenance), 70% nitrous oxide and 30% oxygen. Under a dissecting microscope (20 × magnification) a skin incision was made on one side between the eye and ear. The underlying muscle was retracted down, exposing the semi-translucent skull through which the middle cerebral artery was visible. The middle cerebral artery was exposed by means of a burr-hole craniectomy with removal of underlying dura using a fine curved forceps and then electrocoagulated by bipolar diathermy (Gotti *et al.*, 1989; Welsh *et al.*, 1987). The burr-hole was then filled with bone wax, the area of surgery dusted with Acramide antibiotic and the wound sutured. Animals were allowed to recover from anaesthesia before being returned to holding rooms. Four days later animals were anaesthetized with Saffan (0.9% alphaxolone, 0.3% alphadolone acetate; 300 mg kg^{-1}, i.v.) and the brains fixed via transcardiac perfusion with 10% formalin. Immediately following perfusion, the brains were removed and placed in 10% formalin solution for 48 h. Brains were then processed by successive immersion in 70% (30 min), 90% (2 h) and 100% ethanol (4 × 1.5 h) followed by CNP30 (3 × 1 h) and finally embedded in molten paraffin wax. Coronal sections (3 μm) were cut on a microtome at 300 μm intervals throughout the length of the brain. Sections were dried overnight at 35°C and then stained with haematoxylin and eosin. Slides were coded and the area of infarction (mm^2) in adjacent coronal sections was measured with the image analyser. The volume of infarction (mm^3) was then computed with the trapezoidal rule. In this model the area of infarction is confined mainly to the tempero-parietal cortex and no gross behavioural changes are observed in animals which had undergone permanent unilateral middle cerebral artery occlusion.

Dosing regimens

Gerbil model In one set of animals, GR89696 (3 to 30 μg kg^{-1}, s.c.), GR86014 (1 mg kg^{-1}, s.c.) or GR91272 (1 mg kg^{-1}, s.c.), were administered in two injections immediately before and at 4 h after the 7 min BCO. The ED_{50} value for GR89696 (defined as the dose reducing the neuronal damage by 50%) was determined by the method of Finney (1964). In a second set of animals, GR89696 (100 μg kg^{-1}, s.c.) was administered in three injections 0.5, 4.5 and 8.5 h after the 7 min BCO and GR44821 (10 mg kg^{-1}, s.c.) in three injections 1, 4.5 and 8 h after the 7 min BCO. Saline-injected animals were used as controls. The frequency of dosing was determined by the duration of the sedation produced by these κ-agonists in the gerbil: GR89696 (3 to 300 μg kg^{-1}, s.c.) and GR44821 (10 mg kg^{-1}, s.c.) produced mild to marked sedation which lasted 4 to 5.5 h and 3.5 to 5 h, respectively. GR86014 (1 mg kg^{-1}, s.c.) and GR91272 (1 mg kg^{-1}, s.c.) both produced marked sedation which lasted for 4 to 5 h. In experiments investigating the actions of the opioid antagonist, naltrexone (10 mg kg^{-1}, s.c.), this drug was administered 30 min prior to each injection of GR89696.

Mouse model GR89696 was administered at 300 μg kg^{-1}, s.c. every 4 h after occlusion for the first 24 h and then three times daily for the next three days. In some studies the initial dose was given at 5 min after occlusion and in other experiments at 6 h after occlusion. The frequency of dosing was determined by the duration of the sedation produced in this species: GR89696 (300 μg kg^{-1}, s.c.) produced marked sedation which lasted for 4 h. MK801 was administered at 300 μg kg^{-1}, s.c. at 5 min, 6 h and 18 h after occlusion on the first day and then twice daily for the next three days (Benavides *et al.*, 1989). Saline-injected animals served as controls and underwent middle cerebral artery occlusion.

Drugs

GR44821 (1-[(3,4-dichlorophenyl) acetyl]-2-[(3-oxo-1-pyrrolidinyl)methyl]piperidine maleate), GR86014 (2-[(3,4-dichlorophenyl) acetyl]-1,2,3,4-tetrahydro-1-(1-pyrrolidinyl-methyl)-5-isoquinolinol maleate)), GR89696 (methyl 4-[(3,4-dichlorophenyl)acetyl]-3-(1-pyrrolidinylmethyl)-1-piperazinecarboxylate fumarate), GR91272 (5-[3,4-dichlorophenyl)-acetyl]-4,5,6,7-tetrahydro-4-[(3-hydroxy-1-pyrrolidinyl)-methyl]furo[3,2-c]pyridine hydrochloride) and MK801 ((+)-5-methyl-10,11-dihyro-5H-dibenzo[a,d]-cyclohepten-5,10-imine maleate) were synthesized in the Department of Medicinal Chemistry, Glaxo Group Research. Naltrexone hydrochloride was purchased from the Sigma Chemical Company.

Results

Transient bilateral carotid artery occlusion in the Mongolian gerbil

In the Mongolian gerbil model, administration of GR89696 (3 to 30 μg kg^{-1}, s.c., n = 11–29), immediately before and at 4 h after insult, produced a dose-dependent reduction in the hippocampal CA_1 neuronal necrosis resulting from a 7 min BCO (Figure 1); the ED_{50} value (95% confidence limits) for GR89696 was 11 (7–18) μg kg^{-1}, s.c. Similar results were obtained by pretreatment with two other κ-agonists, GR86014 (1 mg kg^{-1}, s.c.) and GR91272 (1 mg kg^{-1}, s.c.); hippocampal CA_1 neuronal cell areas (mean ± s.e.mean) were: sham-operated, 336 ± 12 μm^2 (n = 22); BCO, 210 ± 28 μm^2 (n = 22); BCO + GR86014, 354 ± 8 μm^2 (n = 10; $P < 0.05$ compared to BCO group); BCO + GR91272, 303 ± 14 μm^2 (n = 12; $P < 0.05$ compared to BCO group). The opioid receptor antagonist, naltrexone (10 mg kg^{-1}, s.c.) had no significant neuroprotective effect on its own (n = 13) but completely prevented the action of GR89696 (n = 13; Figure 2).

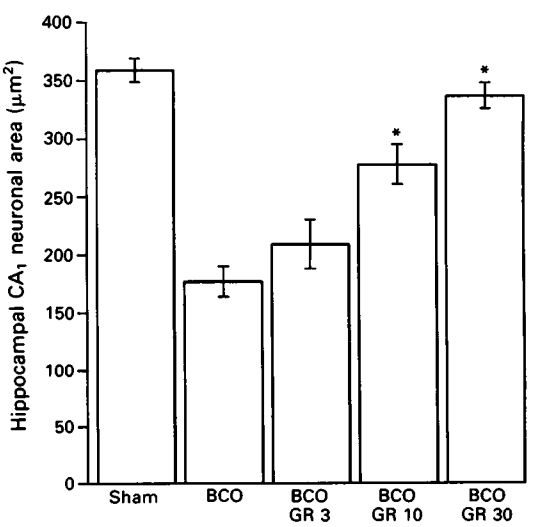

Figure 1 Dose-dependent neuroprotective effects of the κ-opioid receptor agonist GR89696 (GR) in the Mongolian gerbil model of cerebral ischaemia. Gerbils were subjected to a 7 min bilateral carotid occlusion (BCO). Drugs were administered immediately before and at 4 h after occlusion. Doses given are in μg kg^{-1}, s.c. Columns show mean values and s.e.mean indicated by vertical bars. * $P < 0.05$, significantly different from the BCO group (Mann-Whitney test).

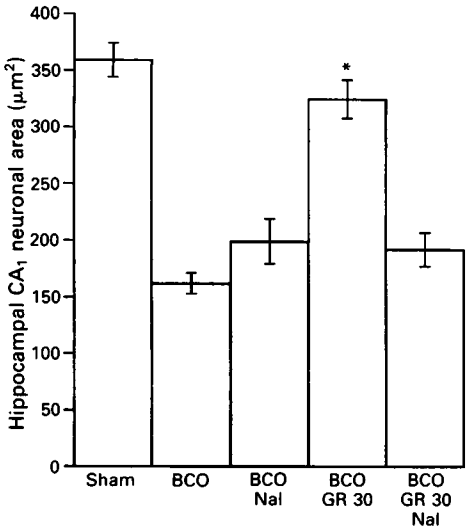

Figure 2 Antagonism by naltrexone (Nal) of the neuroprotective effect of GR89696 (GR) in the Mongolian gerbil model of cerebral ischaemia. GR89696 (30 $\mu g\,kg^{-1}$, s.c.) was administered immediately before and at 4 h after a 7 min bilateral carotid occlusion (BCO). Naltrexone (10 $mg\,kg^{-1}$, s.c.) was administered 30 min prior to each injection of GR89696. Columns show mean values and s.e.mean indicated by vertical bars. * $P < 0.05$, significantly different from the BCO group (Mann-Whitney test).

In a second group of animals, repeated post-treatment, initiated at 30 min post-insult with GR89696 (100 $\mu g\,kg^{-1}$, s.c.; $n = 13$), or initiated at 1 h post-insult with GR44821 (10 $mg\,kg^{-1}$, s.c.; $n = 20$), produced complete protection against the neuronal cell loss resulting from a 7 min BCO (Figure 3).

Permanent unilateral middle cerebral artery occlusion in the mouse

In the mouse middle cerebral artery occlusion model, repeated dosing with GR89696 (300 $\mu g\,kg^{-1}$, s.c.), administered at 5 min, 4, 8, 12, 16, 20, 24 h after occlusion on the first day and then three times daily for the next three days produced a 50% reduction in cerebrocortical infarct volume (infarct volumes were: $8.7 \pm 0.6\,mm^3$, $n = 26$ and $4.3 \pm 0.5\,mm^3$, $n = 30$, for the saline- and GR89696-treated groups, respectively; Figure 4a). Prolonged administration of GR89696 was essential for neuroprotection, as GR89696 produced only a 5% reduction in infarct volume when the dosing covered only the first 16 h ($n = 9$). The neuroprotective effect of GR89696 was slightly greater than that produced by repeated administration of the N-methyl-D-aspartate (NMDA) receptor antagonist MK801 (39% reduction in infarct volume; infarct volumes were: $7.7 \pm 0.6\,mm^3$, $n = 45$ and $4.7 \pm 0.6\,mm^3$, $n = 44$, for the saline- and MK801-treated groups, respectively; Figure 4b).

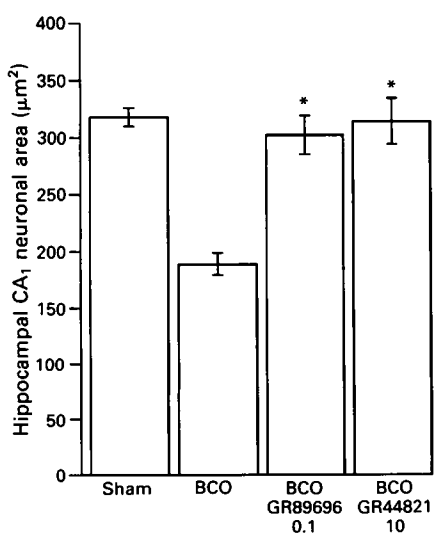

Figure 3 Effect of post-treatment with GR89696 (100 $\mu g\,kg^{-1}$, s.c.) and GR44821 (10 $mg\,kg^{-1}$, s.c.) in the Mongolian gerbil model of cerebral ischaemia. Drugs were administered in three injections at 0.5, 4.5 and 8.5 h (GR89696) or at 1.0, 4.5 and 8.0 h (GR44821) after a 7 min bilateral carotid occlusion (BCO). Columns show mean values and s.e.mean indicated by vertical bars. * $P < 0.05$, significantly different from the BCO group (Mann-Whitney test).

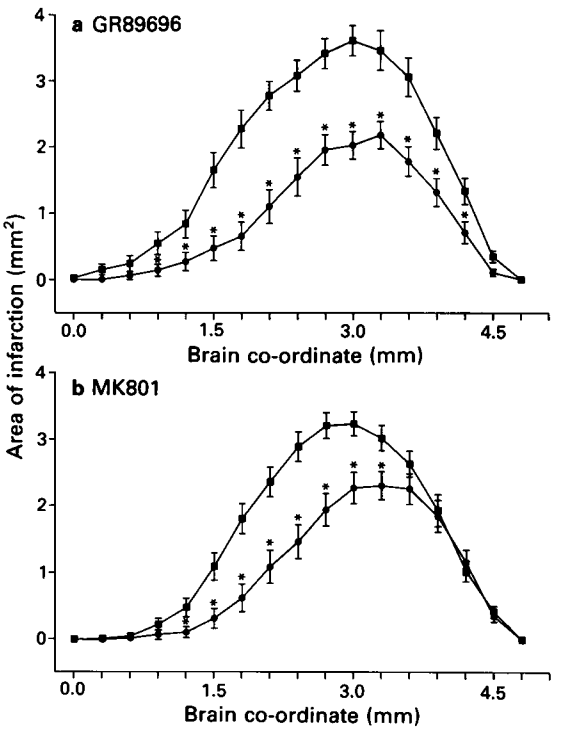

Figure 4 Neuroprotective effect of GR89696 (a) and MK801 (b) in the mouse middle cerebral artery occlusion model. GR89696 (300 $\mu g\,kg^{-1}$, s.c.) was administered at 4 h intervals for the first 24 h and then three times daily for the next three days. Dosing started at 5 min after occlusion. MK801 (300 $\mu g\,kg^{-1}$, s.c.) was administered at 5 min, 6 and 18 h after occlusion on the first day and then twice daily for the next three days. Data points represent area of infarct (mm^2) in adjacent brain sections for saline-treated animals (■) and drug-treated animals (●); s.e.mean indicated by vertical bars. * $P < 0.05$, significantly different from the saline-treated group (Mann-Whitney test).

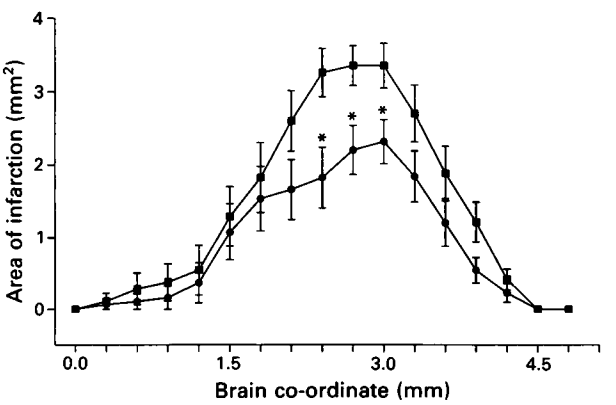

Figure 5 Neuroprotective effect of GR89696 (300 $\mu g\,kg^{-1}$, s.c.) in the mouse middle cerebral artery occlusion model when the initiation of dosing on the first day is delayed for 6 h. Data points represent area of infarct (mm^2) in adjacent brain sections for saline-treated animals (■) and drug-treated animals (●); s.e.mean indicated by vertical bars. * $P < 0.05$, significantly different from the saline-treated group (Mann-Whitney test).

Importantly, GR89696 produced a significant 35% reduction in infarct volume when the initiation of dosing on the first day was delayed for 6 h; infarct volumes were: $7.0 \pm 1.0\,mm^3$, $n = 17$ and $4.6 \pm 0.9\,mm^3$, $n = 18$, for the saline- and GR89696-treated groups, respectively (Figure 5).

Discussion

The results indicate that κ-agonists exert neuroprotective effects in both global and focal models of cerebral ischaemia. More importantly, in both models the compounds are effective when administered post-insult. In the Mongolian gerbil model, the κ-agonists were effective when the start of dosing was delayed for 30 min or 1 h. Interestingly, the non-competitive NMDA receptor antagonist, MK801, has been reported to be neuroprotective when dosing is delayed for 24 h after occlusion in this model (Foster *et al.*, 1987). Experiments with longer post-treatment times were not performed with κ-agonists in the gerbil. In the mouse middle cerebral artery occlusion model, the κ-agonist, GR89696, was efficacious when the start of dosing was delayed for 6 h after occlusion. Interestingly, the NMDA receptor antagonist SL820715 is only effective at time points up to 3 h post-occlusion in the mouse middle cerebral artery occlusion model (Benavides *et al.*, 1989), suggesting that the time window for therapeutic intervention with κ-agonists in this focal cerebral ischaemia model may be superior to NMDA receptor antagonists, although a direct comparison was not made in the present study. The requirement for prolonged dosing with GR89696 in this model is similar to that of SL820715 which becomes more efficacious as the dosing period is increased to cover the first 36 h (Benavides *et al.*, 1989).

κ-Agonists are effective in the gerbil model at doses approximately 100 fold greater than antinociceptive ED_{50} values as determined in the mouse (Hayes *et al.*, 1990). Unfortunately, an antinociceptive test is not available in the gerbil and thus a direct comparison of potencies in this species cannot be made. However, it is important to stress that the neuroprotective action of GR89696 was prevented by naltrexone, indicating that the effect was mediated by activation of an opioid receptor. In the mouse middle cerebral artery occlusion model, lower doses of GR89696 were not tested and thus again an effective comparison between neuroprotective and antinociceptive doses cannot be made. At the doses which are neuroprotective in both gerbil and mouse, κ-agonists produce mild to marked sedation.

In the gerbil model, the opioid receptor antagonist, naltrexone, had no significant neuroprotective effect when administered on its own. This contrasts with published data where this antagonist has been reported to decrease neurological deficit and enhance survival time in a focal cerebral ischaemia model in the cat (Baskin *et al.*, 1985), although in this latter study an effect on infarct size was not observed. Assessments of neurological function were not made in the present study.

The mechanism of action for the neuroprotective effect of κ-agonists is not fully understood, but both neuronal and non-neuronal effects could be involved. It is well established that the excitatory amino acid neurotransmitter, glutamate, plays an important role in ischaemia-induced neurodegeneration and that during ischaemia, synaptic levels of glutamate increase and activate excitatory amino acid receptors. Activation of these receptors leads to a chain of events which results in excessive calcium entry into the neurone and eventual cell death (Meldrum *et al.*, 1985). κ-Agonists may modulate the action of glutamate at presynaptic sites by inhibiting its release or at postsynaptic sites by reducing excitatory amino acid receptor-evoked excessive calcium entry. Recently, κ-agonists have been shown to inhibit glutamate release from rat cerebrocortical slices (Lambert *et al.*, 1991) and to decrease glutamate-stimulated calcium entry into rat cortical cell cultures (DeCoster *et al.*, 1991). However, in our own studies (P.J. Birch, unpublished observations), GR89696 at concentrations up to 1 μM failed to reduce NMDA-induced or anoxia/hypoglycaemia-induced CA_1 neuronal cell loss in a gerbil hippocampal slice preparation, an *in vitro* system in which MK801 is effective (Harrison *et al.*, 1990). This suggests that κ-agonists do not act directly on hippocampal CA_1 neurones to produce their anti-ischaemic effect in the gerbil. κ-Agonists are known to exert anticonvulsant actions (Vonvoigtlander *et al.*, 1987; Tortella *et al.*, 1990) and it has been reported that this effect may involve an interaction between the κ-receptor and the strychnine-insensitive glycine site allosterically linked to the NMDA receptor (Singh *et al.*, 1990). Interestingly, it has been suggested that the anticonvulsant action of κ-agonists may result from a presynaptic inhibition of excitatory amino acid release (Singh *et al.*, 1990) and this could account for the neuroprotection observed in the gerbil.

It has been suggested recently (Corbett *et al.*, 1990) that the neuroprotective effect of MK801 in the gerbil BCO model is due to a drug-related drop in brain temperature. Although high doses of κ-agonists do not decrease body temperature in the gerbil or mouse (P.J. Birch, unpublished observation), brain temperature was not measured or controlled in the present study. κ-Agonists are known to produce a marked diuresis in rodents and such an effect has been suggested to account for the anti-oedematous action of these compounds in rat and cat cerebral ischaemia models (Silvia & Tang, 1986; Silvia *et al.*, 1987). Although no overt diuresis could be detected in the gerbil with doses of GR89696 up to $100\,\mu g\,kg^{-1}$, s.c., this compound produced diuresis in the mouse in the dose range 1 to $100\,\mu g\,kg^{-1}$, s.c. (P.J. Birch, unpublished observations). Hence, an anti-oedema effect may explain in part the neurprotection observed in the focal ischaemia model, although a neuroprotective action of κ-agonists has been demonstrated in rat cortical cell cultures (De Coster *et al.*, 1990) suggesting a neuronal mechanism could also occur. κ-Agonists may also modulate cerebral blood flow, for instance, U50488H has been reported to attenuate post-ischaemic hypoperfusion in a mouse brain injury model (Hall *et al.*, 1987). This has not been assessed in the present study.

In conclusion, κ-opioid receptor agonists are effective neuroprotective agents in both global and focal cerebral ischaemia models and are active when administered post-insult. Although the mechanism of action is as yet undefined, they represent another class of compound which should be considered for clinical evaluation in stroke.

We wish to thank Mrs R. Sargent, Mrs S.M. Harrison and Mr. L. Fernandez for their excellent technical assistance. We also acknowledge the help provided by Mr B. Read and Mr J. Cook from the Department of Histopathology, Glaxo Group Research.

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(Received January 25, 1991
Revised March 13, 1991
Accepted March 18, 1991)