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Neuroprotective effects of 7-nitroindazole in the gerbil model of global cerebral ischaemia

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Abstract

To evaluate the role played by nitric oxide in global cerebral ischaemia we examined the effects of 7-nitroindazole and a sodium salt of 7-nitroindazole (inhibitors of neuronal nitric oxide (NO) synthase) and N^G-nitro-L-arginine methyl ester (a more general inhibitor of NO synthase) in the gerbil model of cerebral ischaemia. Four experiments were carried out. In the first experiment, animals were either sham-operated, subjected to 5 min bilateral carotid occlusion (BCAO) or administered 7-nitroindazole or N^G-nitro-L-arginine methyl ester immediately after occlusion followed by three further doses at 3, 6 and 24 h post-occlusion. In the second experiment, we examined the effects of a sodium salt of 7-nitroindazole, which is more soluble than 7-nitroindazole, using the same protocol. In the third experiment, the effects of the sodium salt of 7-nitroindazole administered at 10 mg/kg at 0, 3, 6, 24, 27, 30, 33, 52, 55, 72, 75 and 78 h post-occlusion or at 0.05 mg/h for 72 h via mini-pumps were evaluated. In separate experiments, we examined the effects of three reference compounds dizocilpine (MK-801), 2,3-dihydroxy-6-nitro-7-sulphamoyl-benz(*F*)-quinoxaline (NBQX) and eliprodil using the same model. Extensive neuronal death was observed in the CA1 layer of the hippocampus in 5 min bilateral carotid occluded animals 5 days after surgery. Both 7-nitroindazole and N^G-nitro-L-arginine methyl ester provided significant neuroprotection ($P < 0.01$) against this neuronal death. The sodium salt of 7-nitroindazole showed no protection when administered up to 12 times post-occlusion, but did provide significant ($P < 0.01$) neuroprotection when administered via mini-pump. The neuroprotection was similar to that provided by MK-801 and eliprodil, but not as good as that observed with NBQX. These results indicate that nitric oxide plays a role in ischaemic cell death and that selective neuronal nitric oxide synthase inhibitors can protect against ischaemic brain damage.

Keywords: Cerebral ischemia; 7-Nitroindazole; Nitric oxide (NO); Hippocampus; (Mongolian gerbil)

1. Introduction

Cerebral ischaemia causes a selective pattern of neurodegeneration (Kirino, 1982; Crain et al., 1988; Ginsberg and Busto, 1989). Several investigators have examined the effects of *N*-methyl-D-aspartate (NMDA) receptor antagonists and α-amino-3-hydroxy-5-methyl-4-isoazole propionate (AMPA) receptor antagonists at preventing this ischaemia-induced damage (Meldrum and Garthwaite, 1990; Sheardown et al., 1990; McCulloch, 1992; Bullock et al., 1990; O'Neill et al., 1995a). More recent studies have examined the role played by nitric oxide in cerebral ischaemia (Choi, 1993; Caldwell et al., 1994; Nagafuji et al., 1995b). It has been shown that activation of the NMDA receptor activates NO synthase, which leads to excess production of NO· (Garthwaite et al., 1989; Moncada et al., 1991). High concentrations of NO· are toxic and interact with O_2^- to produce the highly toxic peroxynitrite anion ($ONOO^-$) (Beckman et al., 1990; Traystman et al., 1991). For this reason, nitric oxide synthase inhibitors have been examined as possible neuroprotective agents (Nowicki et al., 1991; Buisson et al., 1993; Caldwell et al., 1994; Nagafuji et al., 1995a,b). N^G-Nitro-L-arginine has shown neuroprotective effects in global (Caldwell et al., 1994) and focal ischaemia (Nowicki et al., 1991). Protective effects with N^G-nitro-L-arginine methyl ester in global (Caldwell et al., 1995) and focal ischaemia have also been reported (Buisson et al., 1993). However, other investigators report that NO synthase inhibitors enhance damage in global (Weissman et al., 1992) and focal ischaemia (Dawson et al., 1992; Yamamoto et al., 1992). The ability of nitric oxide synthase inhibitors to increase blood pressure due to their blockade of NO in the vascular endothelia could influence outcome after ischaemia (Moncada et al.,

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1991; Caldwell et al., 1994). It has also been suggested that neuronal NO production contributes to the development of ischaemic brain necrosis while endothelial NO may protect brain tissue by increasing ischaemic regional cerebral blood flow (Huang et al., 1994). Further support for the role of neuronal NO in ischaemia has come from experiments which indicated a reduction in infarct volume after middle cerebral artery occlusion in mice deficient in neuronal NO synthase compared with normal mice (Huang et al., 1994). Therefore, selective inhibition of neuronal NO synthase may offer new possibilities for the treatment of cerebral ischaemia.

Recently, it has been reported that 7-nitroindazole is a specific inhibitor of neuronal NO synthase (Babbedge et al., 1993). 7-Nitroindazole produces a potent inhibition of rat cerebellar nitric oxide synthase with an IC_{50} of 0.9 ± 0.1 μM (Babbedge et al., 1993). The compound also exhibits anti-nociceptive activity in the mouse without increasing blood pressure (Moore et al., 1993a,b). Preliminary investigations in the rat have indicated that 7-nitroindazole inhibits NO synthase without any effects on blood pressure and is neuroprotective in focal ischaemia (Yoshida et al., 1994a,b). Clearly, further studies are necessary to examine the effects of this compound in models of global cerebral ischaemia and in models where blood flow is interrupted transiently.

In the present studies, we have evaluated the effects of 7-nitroindazole (20 or 40 mg/kg i.p.) or N^G-nitro-L-arginine methyl ester (10 mg/kg i.p.) immediately after occlusion followed by three further doses (10, 20 or 5 mg/kg, respectively) at 3, 6 and 24 h post-occlusion in the gerbil model of transient global cerebral ischaemia. Using a similar protocol we examined the effects of a sodium salt of 7-nitroindazole administered immediately, 3, 6 and 24 h post-occlusion. As the sodium salt of 7-nitroindazole is more soluble, we compared the effects of administering the compound 12 times post-occlusion to the effects observed when the sodium salt was administered via mini-pumps. Finally we have also examined the effects of MK-801 (an NMDA receptor antagonist), eliprodil (a polyamine site antagonist) and NBQX (an AMPA receptor antagonist) and compared the results with those observed with NO synthase inhibitors.

2. Materials and methods

2.1. 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. They were delivered to the laboratory at least 1 week before commencement of experiments and housed five per cage. The animals were maintained at a constant temperature of 21 ± 1°C and 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 litre/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, taking care not to damage the vagus or sympathetic nerves running close by. In animals to be rendered ischaemic, both carotid arteries were clamped for 5 min. At the end of the occlusion blood flow was re-established. In sham-operated animals, the arteries were exposed but not occluded. The wound was then sutured and the animals allowed to recover. The temperature was maintained at 37°C throughout surgery using a 'K-temp' temperature controller/heating pad (International Market Supply, Chershire, UK) and rectal temperatures were measured. The animals were allowed to recover in a thermacage (Beta Medical and Scientific, UK) which consisted of a 4-compartmental chamber in which the environmental temperature was maintained at 28°C and rectal temperatures were monitored every 30 min for 6 h post-occlusion.

2.2. Experimental design

To examine the effects of N^G-nitro-L-arginine methyl ester 7-nitroindazole and a sodium salt of 7-nitroindazole in the gerbil model of cerebral ischaemia three experiments were carried out. In the first experiment, 7-nitroindazole (RBI, Semat Technical, St. Albans, UK) was dissolved in sesame oil and administered at 20 or 40 mg/kg i.p. immediately after occlusion followed by three further doses of 10 or 20 mg/kg at 3, 6 and 24 h post-occlusion. N^G-Nitro-L-arginine methyl ester (RBI, Semat Technical, St. Albans, UK) was dissolved in 0.89% saline and administered at 10 mg/kg i.p. immediately after occlusion followed by 5 mg/kg i.p. at 3, 6 and 24 h post-occlusion.

In the second experiment, a sodium salt of 7-nitroindazole (Calbiochem-Novabiochem, Nottingham, UK) was dissolved in 0.89% saline and administered at 10 or 20 mg/kg i.p. immediately followed by 5 or 10 mg/kg i.p. at 3, 6 and 24 h post-occlusion.

In the third experiment, the sodium salt of 7-nitroindazole was administered at 10 mg/kg i.p. immediately, 3, 6, 24, 27, 30, 33, 52, 55, 72, 75 and 78 h post-occlusion. A further group of animals had two alzet (model 1003D) osmotic mini-pumps (Charles River, UK) which pump at 1 μl/h for 72 h inserted into the peritoneal cavity. Each pump when full contained 2.5 mg of the sodium salt of 7-nitroindazole in 100 μl. As each animal had two pumps, each of which pumped 0.025 mg/h, this allowed the sodium salt of 7-nitroindazole to be administered at 0.05 mg/h for 72 h.

In the final experiment, the effects of MK-801 administered at 2 mg/kg i.p., eliprodil administered at 20 mg/kg i.p. immediately followed by 10 mg/kg at 3 and 6 h

post-occlusion and NBQX administered at 30 mg/kg i.p. 0, 3 and 6 h post-occlusion were examined.

2.3. Histology

5 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. 5 μm coronal sections were taken at 1.5, 1.7 and 1.9 mm caudal to the bregma in the anterior hippocampus 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 neuronal density per mm CA1 hippocampus.

2.4. Statistics

Statistical analysis of histological data was carried out using analysis of variance (ANOVA) followed by Student's *t*-test with Bonferroni corrections using $P < 0.05$ as the level of significance.

3. Results

No change in rectal temperature was observed with any of the compounds used. 5 μm sections taken 1.5–1.9 mm caudal to the bregma in the anterior hippocampus were examined under a microscope with grid lines. The CA1 pyramidal neurones were found to be degenerated in the 5 min occluded animals (Fig. 1 and Fig. 2). The neuronal death involved nearly all the neurones and this neurodegeneration was not evident in any other forebrain region. The pyramidal cell density was counted at three different stereotaxic levels in the CA1 region of the hippocampus and the results expressed as means ± S.E.M. neuronal density per 1 mm CA1. Both doses of 7-nitroindazole provided significant protection against the ischaemia-induced cell death in the CA1 region of the hippocampus (Fig. 1.). N^G-Nitro-L-arginine methyl ester also provided neuroprotection (23%) which was greater than that of 7-nitroindazole (Fig. 1).

In the second experiment, the lower dose of the sodium salt of 7-nitroindazole failed to provide any neuroprotection. The higher doses provided slight protection, but this did not reach significance (Fig. 2).

In the third experiment, the sodium salt of 7-nitroindazole (10 mg/kg i.p.) when administered at 12 time intervals post-occlusion provided some neuroprotection, but this did not reach significance (Fig. 3a). However, the compound did provide significant neuroprotection (26%), when administered via mini-pumps over a 72 h period (Fig. 3b).

The protection we obtained with these NO synthase inhibitors is similar to the results we have observed with 2 mg/kg of the NMDA antagonist MK-801 (24%) and

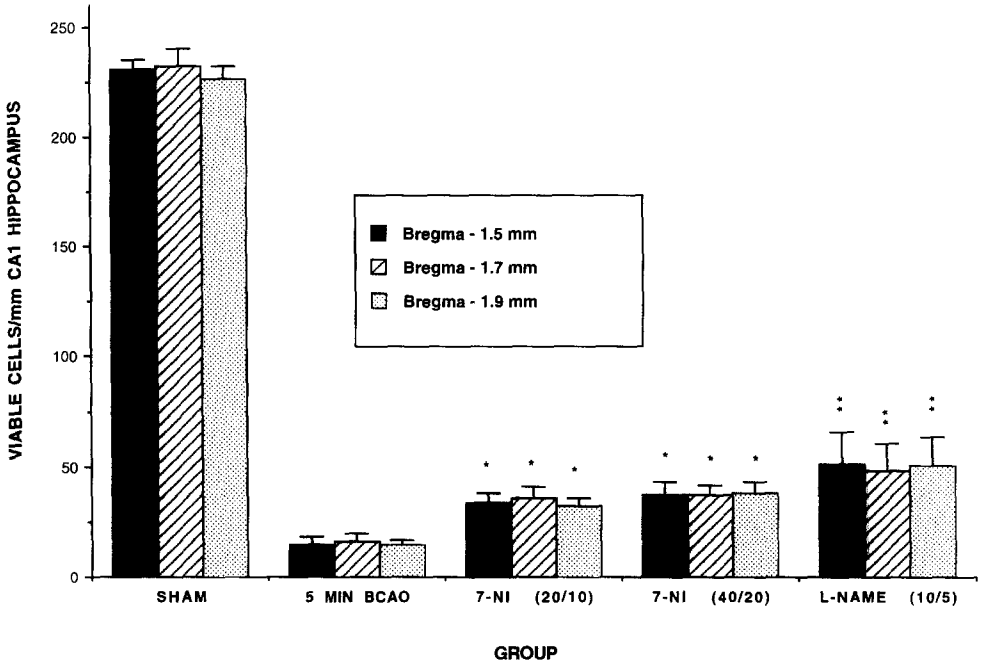

Fig. 1. The effects of 7-nitroindazole or N^G-nitro-L-arginine methyl ester on the neuronal density in the CA1 region of the hippocampus 5 days after 5 min bilateral carotid artery occlusion. 7-Nitroindazole was administered at 20 or 40 mg/kg immediately post-occlusion followed by three further doses of 10 or 20 mg/kg at 3, 6 and 24 h post-occlusion. N^G-Nitro-L-arginine methyl ester was administered at 10 mg/kg immediately post-occlusion, followed by 5 mg/kg at 3, 6 and 24 h. Results are expressed as means ± S.E.M. viable cells/mm CA1 hippocampus ($n = 8$ animals per group). 5 min bilateral carotid artery occlusion caused a severe loss in neuronal cells in the CA1 region ($P < 0.001$). Both doses of 7-nitroindazole provide significant neuroprotection ($P < 0.05$) against the ischaemia-induced cell death, while N^G-nitro-L-arginine methyl ester provided greater protection ($P < 0.01$). Student's *t*-test.

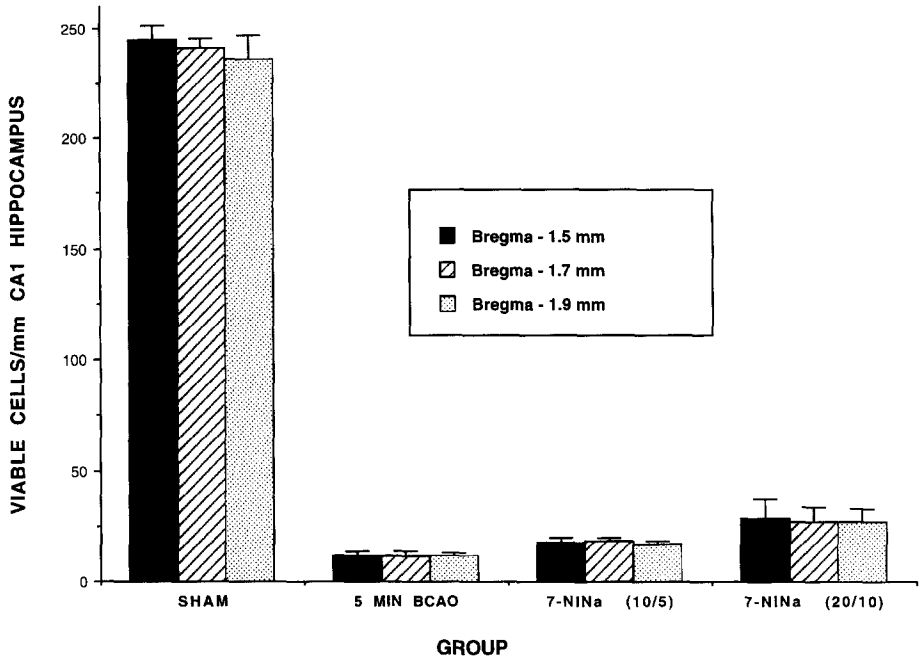

Fig. 2. The effects of a sodium salt of 7-nitroindazole on the neuronal density in the CA1 region of the hippocampus 5 days after 5 min bilateral carotid artery occlusion. The sodium salt of 7-nitroindazole was administered at 10 or 20 mg/kg immediately post-occlusion followed by three further doses of 5 or 10 mg/kg at 3, 6 and 24 h post-occlusion. 5 min bilateral carotid artery occlusion caused a severe loss in neuronal cells in the CA1 region ($P < 0.001$). The lower dose of the sodium salt of 7-nitroindazole did not provide any protection. The higher dose provided some neuroprotection, but this did not reach significance.

eliprodil 20 mg/kg followed by 2 doses of 10 mg/kg at 3 and 6 h (Fig. 4). However, 30 mg/kg of NBQX administered immediately, 3 and 6 h post-occlusion provided greater neuroprotection (43%) in the same model (Fig. 4). Statistical analysis indicated that NBQX provided significantly more protection than the low doses of 7-nitroindazole, but although NBQX provided greater protection than the high dose of 7-nitroindazole and 7-NINa this failed to reach significance.

4. Discussion

Recent studies have examined the role of NO and NO synthase in cerebral ischaemia and other cerebrovascular disease states (Choi, 1993; Caldwell et al., 1994; Dawson, 1995; Yoshida et al., 1994a,b). Ischaemia is known to release glutamate, which acts on the NMDA and AMPA receptors to initiate a cascade of calcium-mediated toxicity, lipid peroxidation and free radical production (Siesjö, 1992a,b; O'Neill et al., 1995b). It has recently been shown that activation of the NMDA receptor activates NO synthase, which leads to excess production of NO· (Moncada et al., 1991; Garthwaite et al., 1989). Therefore, NO synthase inhibitors have been examined as possible neuroprotective agents (Nowicki et al., 1991; Dawson et al., 1992; Caldwell et al., 1994; Yoshida et al., 1994a,b). In the present study, we have examined the effects of 7-nitroindazole and N^G-nitro-L-arginine methyl ester in the gerbil model of global cerebral ischaemia. We have previously shown that low doses of N^G-nitro-L-arginine methyl ester can attenuate ischaemia-induced increases in lipid peroxidation in the gerbil hippocampus (Caldwell et al., 1995). In the present study, we have shown that similar doses of N^G-nitro-L-arginine methyl ester protect (23%) against ischaemia-induced CA1 pyramidal cell death. The doses used are similar to those used in other recent studies that have shown protective effects with N^G-nitro-L-arginine methyl ester (Buisson et al., 1993; Dawson et al., 1994; Quast et al., 1995). It has been suggested that lower doses of NO synthase inhibitors may inhibit the cerebral NO synthase with little effect on the endothelial enzyme and this may explain why low doses have given protective effects and higher doses have enhanced the damage (Buisson et al., 1993). We have previously reported that 10 mg/kg of N^G-nitro-L-arginine attenuates ischaemia-induced hyperactivity and increases in nitric oxide synthase activity in the gerbil brain (Caldwell et al., 1994). We have also shown that N^G-nitro-L-arginine methyl ester can also attenuate ischaemia-induced hyperactivity and increases in nitric oxide synthase activity in the same model (unpublished observations). However, some investigators have reported no neuroprotection with NO synthase inhibitors (Weissman et al., 1992; Yamamoto et al., 1992) and this may be due to experimental design and the effects of the doses of the inhibitors used on NO in the vascular endothelia (Moncada et al., 1991). Kohno et al. (1995) have recently reported that intraventricular administration of

N^G-nitro-L-arginine or N^G-nitro-L-arginine methyl ester can protect against 5 min BCAO in the gerbil. They have also shown that intraventricular administration of N^G-nitro-D-arginine or N^G-nitro-D-arginine methyl ester caused no effect on the damage produced.

It has also been suggested that neuronal NO production contributes to the development of ischaemic brain necrosis while endothelial NO may protect brain tissue by increasing ischaemic regional cerebral blood flow (Huang et al., 1994). Therefore, we have examined selective inhibition of neuronal NO synthase with 7-nitroindazole in the gerbil model of global cerebral ischaemia. It has been reported that 7-nitroindazole produces a potent inhibition of rat cerebellar NO synthase with an IC_{50} of 0.9 ± 0.1 μM (Babbedge et al., 1993). Recent studies in the rat have indicated that 7-nitroindazole inhibits NO synthase without any effects on blood pressure and is neuroprotective in focal ischaemia (Dalkara et al., 1994; Yoshida et al., 1994a,b). We have now shown that 7-nitroindazole is protective (17.5%) in the gerbil model of global cerebral ischaemia. The sodium salt of 7-nitroindazole failed to provide significant protection when administered using the same protocol. However, the sodium salt of 7-nitroindazole did provide a substantial protective effect (26%) when administered via mini-pumps. A possible explanation for this could be the fact that 7-nitroindazole has a short half-life compared to N^G-nitro-L-arginine methyl ester and only when it is released slowly from oil or continuously infused via a mini-pump can significant levels be maintained. This is supported by studies that show that N^G-nitro-L-arginine methyl ester produces long-lasting (> 24 h) antinociception in the mouse (Moore et al., 1991), whereas with 7-nitroindazole the effect is short lived (60 min) (Moore et al., 1993a). It has been shown that N^G-nitro-L-arginine methyl ester is an irreversible inhibitor of brain NO synthase (Dwyer et al., 1991), while 7-

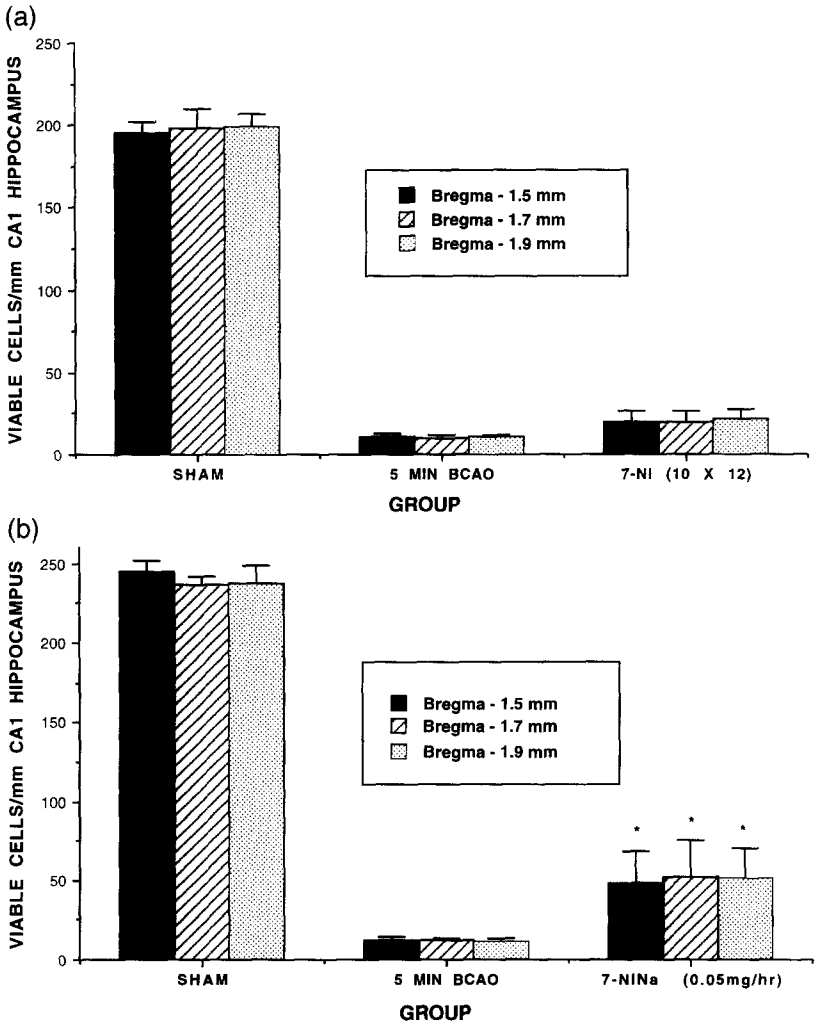

Fig. 3. The effects of a sodium salt of 7-nitroindazole administered at 10 mg/kg i.p. 0, 3, 6, 24, 27, 30, 33, 52, 55, 72, 75 and 78 h post-occlusion (Fig. 3a) or administered at 0.05 mg/h for 72 h via mini-pumps (Fig. 3b). Results are expressed as means ± S.E.M. viable cells/mm CA1 hippocampus ($n = 8$ animals per group). 5 min bilateral carotid artery occlusion caused a severe loss in neuronal cells in the CA1 region ($P < 0.001$). The sodium salt of 7-nitroindazole did not provide any protection when administered 12 times post-occlusion. However, the sodium salt of 7-nitroindazole did provide significant protection ($P < 0.05$) against the ischaemia-induced cell death when administered continuously via mini-pumps. Student's t-test.

nitroindazole competes with L-arginine for the substrate site (Babbedge et al., 1993) and this may explain the short-lived activity observed with 7-nitroindazole (Moore et al., 1993b). Schulz et al. (1995) have recently reported that 7-nitroindazole also protects against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity in mice.

The protection observed in the present studies (17.5% with 40 mg/kg 7-nitroindazole, 23% with 10 mg/kg N^G-nitro-L-arginine methyl ester, and 26% with the sodium salt of 7-nitroindazole) is similar to the protection we have observed with NMDA receptor antagonists (24% with 2 mg/kg MK-801), polyamine site antagonists (25% with 20 mg/kg eliprodil), but not as good as observed with an AMPA receptor antagonist (43% with 30 mg/kg NBQX) administered post-occlusion (Fig. 4).

There is also new evidence from several studies suggesting that inducible NO synthase may be important in cerebral ischaemia. Murphy and collaborators have reported that inducible NO synthase is regulated by cytokine-activated astrocytes (Borgerding and Murphy, 1995). Studies by Choi and coworkers have shown that induction of astrocytic inducible NO synthase causes potentiation of NMDA-induced neuronal injury in cell cultures (Hewett et al., 1994). It has also been reported that the inducible NO synthase inhibitor, *S*-methylisothiourea sulfate improved survival in rodent models of septic shock (Szabó et al., 1994). Preliminary results from our laboratory have indicated that *S*-methylisothiourea sulfate produces a small, but non-significant, neuroprotective effect in the gerbil model of cerebral ischaemia (O'Neill et al., unpublished results). Iadecola and coworkers have shown that ischaemia-induced inducible NO synthase mRNA expression begins at 12 h and peaks at 48 h in a rat model of focal ischaemia (Iadecola et al., 1995). The inducible NO synthase mRNA expression paralleled the time course of induction of inducible NO synthase activity. The group have also shown that the inducible NO synthase inhibitor aminoguanidine administered for 3 days, beginning 24 h after stroke, reduced the infarct volume by 33% in a rat model of cerebral ischaemia (Iadecola et al., 1995; Zhang et al., 1996).

A number of recent studies have shown that NO synthase activity is increased in the rodent brain after global (Caldwell et al., 1994) and focal cerebral ischaemia (Yoshida et al., 1995). We have also shown that the ischaemia-induced increases in nitric oxide synthase can be prevented by NO synthase inhibitors (Caldwell et al., 1994) and σ receptor ligands (O'Neill et al., 1995a). Other groups have reported increases in NO after global ischaemia using in vivo measurement with a nitric oxide sensor (Niiro et al., 1995). Further support for the role of neuronal NO in ischaemia has come from experiments which indicated that there is a reduction in infarct volume after MCAO in mice deficient in neuronal NO synthase compared with normal mice (Huang et al., 1994).

In conclusion, it is clear that NO does play a role in cerebral ischaemia. In the present studies we have shown that the neuronal nitric oxide synthase inhibitor 7-nitroindazole can protect against ischaemia-induced hip-

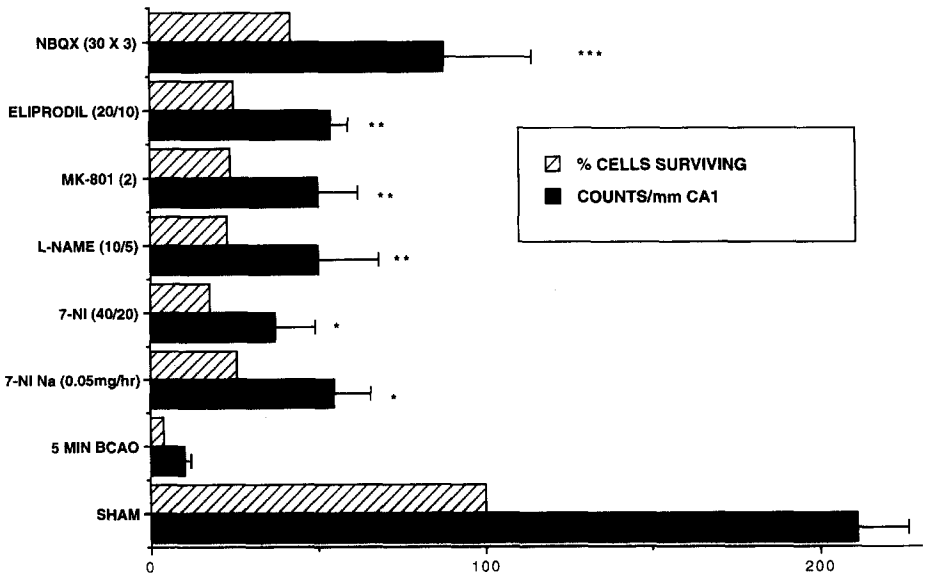

Fig. 4. Comparison of the effects of an NMDA antagonist (MK-801), a polyamine site antagonist (eliprodil) and an AMPA antagonist (NBQX) with the nitric oxide synthase inhibitors (N^G-nitro-L-arginine methyl ester, 7-nitroindazole and a sodium salt of 7-nitroindazole). Results are expressed as means ± S.E.M. viable cells/mm CA1 hippocampus ($n = 8–10$ animals per group). It is clear that all the nitric oxide synthase inhibitors provide similar protection to MK-801 and eliprodil. NBQX provided greater protection in this model. * $P < 0.05$, * * $P < 0.01$, * * * $P < 0.005$, Student's *t*-test.

pocampal damage in the gerbil. Clearly there is a need for more selective neuronal NO synthase inhibitors which may be a useful strategy for the treatment of cerebral ischaemia.

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