Blockade of intracellular actions of calcium may protect against ischaemic damage to the gerbil brain

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1 The brain cytoprotective effects of a putative calcium-associated protein kinase inhibitor, HA1077, as well as a calcium entry blocker nicardipine were evaluated in models of cerebral ischaemia in Mongolian gerbils. Morphological changes characterizing delayed neuronal death of selectively vulnerable CA₁ pyramidal neurones in the hippocampus of the Mongolian gerbil brain occurred 7 days after transient bilateral occlusion of the common carotid arteries.

2 A single injection of HA1077 (1 and 3 mg kg⁻¹, i.p.) 5 min after the occlusion led to a dose-dependent protection of the CA₁ neurones. Repeated administrations of HA1077 (1 and 3 mg kg⁻¹, i.p., twice daily for 7 days post-ischaemia) revealed an increase in the number of normal cells, compared to findings with a single administration.

3 In contrast to HA1077, nicardipine (0.3 and 1 mg kg⁻¹, i.p.) did not reduce neuronal degeneration.

4 HA1077 did not interact with the ion channel within which MK-801 binds, as determined by receptor binding.

5 The calcium ionophore, A23187, caused a tonic contraction in canine cerebral arterial strips. HA1077, but not nicardipine, relaxed the A23187-induced contraction, concentration-dependently.

6 These results suggest that blockade of the intracellular actions of calcium may provide protection against ischaemic damage in the brain.

Keywords: Delayed neuronal death; ischaemia; calcium antagonists; protein kinase; HA1077

Introduction

Kirino (1982) described a gerbil model of forebrain ischaemia, induced by transient occlusion of both common carotid arteries, the result being a reproducible discrete lesion featuring extensive neuronal degeneration in the CA₁ subfield of the hippocampus, in 90% of the experiments. It takes several days for this neuronal damage to become apparent at the light microscopic level, hence this phenomenon was termed 'delayed neuronal death' (Kirino, 1982). A similar temporal profile of neuronal ischaemic damage in rats subjected to transient forebrain ischaemia has been described by Pulsinelli *et al.* (1982). It has been suggested that delayed neuronal death is mediated by the following events: (1) excessive release of glutamate from nerve terminals during the ischaemic period; (2) activation of N-methyl-D-aspartate (NMDA)-type glutamate receptors, and (3) influx of Ca²⁺ into the neurones, probably through NMDA receptor-linked calcium channels. Although factors following these events are not clear, nor is the precise mechanism of the delayed onset of the neuronal damage well understood, both require attention when attempting to design treatment for postischaemic brain damage in man.

The pathological accumulation of intracellular calcium is thought to be one factor that causes or accompanies cell death (Siesjö, 1981). Neuronal cell damage observed after transient cerebral ischaemia was found to correlate strongly with an intracellular accumulation of calcium (DeLeo *et al.*, 1987).

Attention has been given to various calcium modulators for their potential experimental and clinical usefulness in the therapy of disorders related to cerebral ischaemia.

Thus, we examined a novel calcium antagonistic vasodilator HA1077 (1-5-(isoquinolinesulphonyl)-homopiperazine HCl), proposed as a calcium-associated protein kinase inhibitor (Asano *et al.*, 1989), and nicardipine, a calcium entry blocker which prevents ischaemia-induced cell death in hippocampal neurones of Mongolian gerbils.

HA1077 was found to produce a competitive inhibition of Ca²⁺-induced contractions of strips of depolarized rabbit aorta and significantly inhibited phenylephrine-induced contraction in Ca²⁺-free solution (Asano *et al.*, 1987). The contraction in response to adrenoceptor stimulation in Ca²⁺-free solution seems to be due almost entirely to the release of intracellular Ca²⁺ (Fleckenstein, 1977). HA1077 did not alter the developed tension in the guinea-pig left atrium, hence, unlike calcium entry blockers, HA1077 does not have a blocking action on slow myocardial calcium channels (Asano *et al.*, 1987). Cultured vascular smooth muscle cells exposed to HA1077 showed a dose-dependent loss or a decrease in size and length of the actin-containing microfilament structure (Sasaki *et al.*, 1987). Neither verapamil nor diltiazem induced reorganization of the actin microfilament structure. HA1077 potently inhibited calcium-associated protein kinases considered to have an important role in regulation of cellular function (Asano *et al.*, 1989). These results suggest that the mechanism of action of HA1077 differs from that of conventional calcium entry blockers and that its effect may depend on actions on internal sequestering sites.

Methods

Delayed neuronal death study

Male Mongolian gerbils, weighing 55 to 80 g, were placed in the supine position. Under light anaesthesia with ether, both common carotid arteries were exposed through a ventral midline incision and separated carefully from the adjacent vein and sympathetic nerves. The arteries were then clamped with aneurysm clips. After 5 min, the clips were removed and the skin was sutured. Sham-operated animals were treated in the same manner, except for no clamping.

Test drug solution or saline (0.9% w/v NaCl) was injected i.p. 5 min after artery occlusion in the single injection groups. After the single injection, in addition, repeat injection groups

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were given doses of the respective test drug twice daily at intervals of 12 h during the post-ischaemia period.

On day 7 after a 5 min occlusion, the animals used for the histopathological examination were anaesthetized with pentobarbitone and the brains were perfused with a 10% buffered formalin solution given through the left cardiac ventricle. The brains were dissected out and fixed in 10% buffered formalin solution. The hippocampal region was cut coronally into 3 to 4 mm thick slices, embedded in paraffin and processed with the step section technique. The slides were stained with haematoxylin and eosin and cresyl violet.

The pyramidal cells of each CA₁ region were counted under a light microscope at $\times 200$ magnification. The mean number of CA₁ pyramidal neurones per millimetre for both hemispheres, (the neuronal density), was calculated for each group, according to Izumiyama & Kogure (1988).

Vascular relaxation study

Mongrel dogs were anaesthetized with sodium pentobarbitone and exsanguinated from the femoral artery. Basilar arteries were rapidly removed and kept on crushed ice in oxygenated Krebs-Henseleit solution (mm: NaCl 115, KCl 4.7, CaCl₂ 2.5, MgCl₂ 1.2, NaHCO₃ 2.5, KH₂PO₄ 1.2 and dextrose 10.0). Each artery was helically cut into equal strips 20–25 mm long and 1–2 mm wide and the strips were suspended under a resting tension of 1.0 g in a 20 ml tissue bath containing Krebs-Henseleit solution at 37°C; they were aerated continuously with a mixture of 95% O₂ and 5% CO₂. Changes in arterial tension were recorded isometrically through a force-displacement transducer (Nihon Kohden, SB-1T) and a polygraph.

After 60–90 min of equilibration, 40 mM KCl was added to the bath two or three times until successive responses of the tissue remained constant. Helical strips of the basilar artery were contracted with 3×10^{-6} M A23187, and when the contraction had reached a plateau, HA1077 or nicardipine was added.

NMDA receptor antagonism study

After male Sprague-Dawley rats (200–250 g, Charles River) were killed by cervical dislocation their brains were removed and rapidly dissected at 4°C to obtain cerebral cortex and hippocampus. All further procedures were carried out at 4°C. The cerebral cortex and hippocampus were homogenized separately in 30 volumes of 10 mM HEPES buffer (pH 7.4) with a Teflon-glass homogenizer. The homogenate was centrifuged at 48,000 *g* for 15 min. The pellet was resuspended in 30 volumes of distilled water and lysed at 37°C for 20 min followed by centrifugation at 48,000 *g* for 15 min. This process was repeated once more. The resulting pellet was resuspended in 30 volumes of 10 mM HEPES containing 1 mM EDTA and frozen at -80°C until use in binding experiments.

On the day the binding assay was performed, tissue suspensions were thawed, homogenized in 100 volumes (with respect to original tissue weight) of 10 mM HEPES and centrifuged at 48,000 *g* for 15 min. The pellet was resuspended in 10 volumes of 10 mM HEPES buffer. All preparative procedures and binding assays were performed in ultrapure water.

For [³H]-MK-801 binding assays, the tissue preparations were incubated in duplicate with 5 nM [³H]-MK-801 in the presence of various concentrations of unlabelled drugs, 10^{-5} M glutamate and glycine. Incubations were carried out for 60 min at 25°C in a final volume of 1 ml. Nonspecific binding was determined with 10^{-5} M phenylcyclidine. The binding reaction was terminated by filtration under vacuum through Whatman GF/C glass fibre filters. Filters were washed twice with 5 ml of 10 mM HEPES buffer and the retained radioactivity determined using a liquid scintillation counter.

Statistics

The values are given as mean $\pm$ s.e.mean. The significance of difference was calculated by Student's *t* test or Duncann's multiple range test. *P* values of 0.05 or less were considered to represent significant differences.

Drugs

The drugs used were nicardipine HCl (Yamanouchi, Tokyo, Japan), [³H]-MK-801 (New England Nuclear, Boston, MA, U.S.A.) and phenylcyclidine (Sigma, St Louis, MO, U.S.A.).

Results

Delayed neuronal death study

Complete forebrain ischaemia for a period of 5 min resulted in a model that showed consistent neuronal degeneration, in

Figure 1 Light micrographs of coronal sections stained with haematoxylin and eosin and showing morphological changes on day 7 following transient ischaemic insult. Bar represents 20 μm . (a) Pattern of degeneration of pyramidal cells in CA₁ area in the hippocampus of a sham-operated gerbil. (b) Shows significant loss of structural integrity and neuronal death in a saline-treated animal subjected to 5 min bilateral carotid occlusion. Ghost cell spaces, darkly stained shrunken dead cells and cellular debris are characteristic features of delayed neuronal death. (c) Significant protection of pyramidal cells in the hippocampus of a gerbil given repeated injections of HA1077 (3 m, kg⁻¹, i.p., twice daily) during ischaemia.

Table 1 Effects of drug treatment on delayed neuronal death in the hippocampus CA₁ subfield of the gerbil brain

Treatment	Normal cell count (cells mm ⁻¹)	
	Single injection	Repeat injection
Saline	17.8 ± 2.1 (n = 14)	17.4 ± 2.8 (n = 10)
HA1077 1 mg kg ⁻¹	34.4 ± 6.6* (n = 15)	71.3 ± 23.9* (n = 11)
HA1077 3 mg kg ⁻¹	40.0 ± 9.9* (n = 15)	92.0 ± 17.3**† (n = 10)
Nicardipine 0.3 mg kg ⁻¹	29.5 ± 5.7 (n = 10)	
Nicardipine 1 mg kg ⁻¹	28.0 ± 6.4 (n = 10)	

Saline or test drug solution was injected i.p.

Values are mean ± s.e.mean (n = numbers of animals).

* $P < 0.05$; ** $P < 0.01$ vs. each saline treatment group.

† $P < 0.05$ vs. each single injection group.

each animal. The pattern of neurodegeneration involved virtually all pyramidal neurones in the CA₁ areas of both hippocampus on day 7 (Figure 1). Assessment of the hippocampal damage by measurement of the normal pyramidal neurones of the CA₁ region proved to be a convenient and highly consistent approach. In the sham-operated gerbils, the normal neurone count was 213 ± 5 cells mm⁻¹ (n = 11). In the saline-treated animal, the normal cell count was significantly different from sham-operated animals on day 7.

Table 2 Relaxation of A23187-induced contraction by HA1077 in canine basilar arteries

	Tension (mg)	n
HA1077 treatment		
A23187 alone	1605 ± 89	8
+ HA1077 10 ⁻⁶ M	1326 ± 93**	8
+ HA1077 10 ⁻⁵ M	816 ± 89**	8
+ HA1077 10 ⁻⁴ M	681 ± 89**	8
Nicardipine treatment		
A23187 alone	1789 ± 259	3
+ nicardipine 10 ⁻⁶ M	1798 ± 251	3
+ nicardipine 10 ⁻⁵ M	1834 ± 230	3

HA1077 or nicardipine were added to helical strips of canine basilar artery precontracted with 3×10^{-6} M A23187. HA1077 or nicardipine were added after peak tension had been achieved. Values are mean ± s.e.mean. n = numbers of animals.

** $P < 0.01$ by Student's paired *t* test vs. control group.

Figure 2 Effect of HA1077 (○) and MK-801 (●) on [³H]-MK-801 binding in the rat cerebral cortex (a) and hippocampus (b). Each point represents mean value for 2 determinations performed in duplicate. Assays contained 10⁻⁵ M glutamate and glycine.

A single injection of HA1077 (1 and 3 mg kg⁻¹, i.p.) 5 min after bilateral occlusion led to a dose-dependent increase in the normal cell count in the CA₁ region (Table 1). In contrast, nicardipine (0.3 and 1 mg kg⁻¹, i.p.) did not reduce the area of neuronal degeneration in the hippocampus.

Repeat injection of HA1077 (1 and 3 mg kg⁻¹, i.p., twice daily) also significantly reduced nerve cell damage, in a dose dependent manner (Figure 1 and Table 1).

Morphological findings of the repeat saline-treated groups were similar to those of the single saline-treated groups. However, repeat injection of HA1077 (3 mg kg⁻¹, i.p., twice daily) significantly increased the number of normal cells compared to findings in the single injection group. Although there were large differences in the mean values in the HA1077 1 mg kg⁻¹-treated group, the differences were not statistically significant.

Vascular relaxation study

The canine basilar artery contracted with calcium ionophore A23187 responded differently to HA1077 and nicardipine, respectively (Table 2). Cumulative increase in the concentration of HA1077 (10⁻⁶ M to 10⁻⁵ M) added to canine basilar arterial strips contracted by 3×10^{-6} M A23187 resulted in a concentration-dependent relationship for relaxation. Conversely the same procedure in the concentration of nicardipine (10⁻⁶ M to 10⁻⁵ M) had no effect on the contraction produced by A23187.

NMDA receptor antagonism study

HA1077 did not inhibit the specific binding of the NMDA-type receptor antagonist [³H]-MK-801 in the rat cerebral cortex or hippocampus preparations (Figure 2).

Discussion

The present results show that a novel calcium antagonist and vasodilator, HA1077, reduces the incidence of delayed neuronal death in the CA₁ subfield of the gerbil hippocampus following transient bilateral carotid artery occlusion.

The present NMDA receptor antagonism study relied on the high specificity of MK-801 so that a crude homogenate could be used. As shown in Figure 2, HA1077 clearly differed from a noncompetitive NMDA receptor channel blocker, MK-801. HA1077 had no effect on MK-801 binding. This is not indicative of a lack of effect at the NMDA receptor, but a lack of effect on the ion channel within which MK-801 binds, since binding was performed at equilibrium and in the presence of high concentrations of glutamate and glycine. Glutamate (10⁻⁵ M) was included, to activate fully the NMDA receptor and allow full access to the open channel. The glycine level was held high for the same reason. Furthermore, we have used cold MK-801 to displace ³H-labelled MK-801. In a subsequent study, use of another ligand (e.g. TCP) may be preferable.

The accumulation of intracellular calcium is thought to be a major triggering event in the development of tissue damage following ischaemia in the brain (Siesjö, 1981). In attempts to prevent the harmful effects associated with Ca²⁺ influx, a block or decrease the influx of calcium by use of calcium entry blockers has been examined. Some investigators reported that these agents may decrease cerebral damage (Steen *et al.*, 1983; Alps *et al.*, 1988) while no beneficial effect has also been noted (Steen *et al.*, 1984).

Alps *et al.* (1988) administered calcium entry blockers such as nicardipine and nimodipine before 5 min-carotid artery occlusion in the Mongolian gerbil. The overall protection afforded by nicardipine (0.5 mg kg⁻¹, i.p.) was statistically significant. Nimodipine (0.5 mg kg⁻¹, i.p.) was inactive in this

model (Alps *et al.*, 1988). This beneficial effect of pre-ischaemia treatment with nicardipine may be due to a block of the influx of Ca^{2+} . The question of treatment with nicardipine during the post-ischaemic period in gerbils has not been documented. In the present study, nicardipine (0.3 and 1 mg kg^{-1} , i.p.) administered after bilateral occlusion led to no decrease in neurodegeneration. This discrepancy may be related to the different protocols (pre-ischaemia administration vs. post-ischaemia administration). A disturbance in calcium homeostasis was evident in the gerbil hippocampus after a brief transient ischaemia (Sakamoto *et al.*, 1986). We assumed that the intracellular calcium concentration in nerve cells had been raised, when nicardipine was administered after an ischaemic event. Thus, post-ischaemic treatment with nicardipine could not protect against neuronal degeneration.

Calcium ionophore, A23187, transports calcium as a lipid soluble complex through a number of natural and artificial membranes and increases intracellular calcium concentrations (Schaffer *et al.*, 1973). In contrast to nicardipine, HA1077 relaxed the A23187-induced contraction of basilar artery strips. The present result is in agreement with the previous studies which suggested that HA1077, unlike calcium entry blockers, can block the intracellular actions of calcium (Asano *et al.*, 1987; 1989; Sasaki *et al.*, 1987).

Of greater interest is the decrease in brain damage when HA1077 was administered following ischaemia. We suggest

that the brain cytoprotective effect of HA1077 is related to its ability to block the intracellular actions of calcium as discussed above, i.e., HA1077 could block adverse reactions detrimental to the cell triggered by excessive intracellular calcium after ischaemia.

In support of our working hypothesis, we found that HA1077 potently inhibits certain calcium related-protein kinases (Asano *et al.*, 1989). For example, the K_i value of HA1077 for guanosine 3':5'-cyclic monophosphate (cyclic GMP) or adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase was $1.6 \times 10^{-6} \text{ M}$. The concentrations of HA1077 inhibiting agonist-induced vascular contractions were similar to those inhibiting protein kinase. The potent kinase antagonistic effects of HA1077 are likely to be related to the blocking action of intracellular calcium during ischaemia and contribute to the anti-ischaemic effect of HA1077. It should be noted that ischaemia, hypoxia, and NMDA produce increases in cerebellar cyclic AMP or cyclic GMP production (Yoshida *et al.*, 1980; Siesjo, 1981). The involvement of calcium-related protein kinases in the evolution and consequences of cerebral ischaemia remains to be determined.

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