British Journal of Pharmacology (1995) 115, 1439-1446

Reduction by lifarizine of the neuronal damage induced by cerebral ischaemia in rodents

[1]B.J. Alps, C. Calder, A.D. Wilson, D.E. McBean & J.M. Armstrong

Department of Pharmacology, Syntex Research Centre, Research Avenue South, Heriot Watt University Research Park, Riccarton, Edinburgh EH14 4AP

1 The objective of this study was to evaluate the broad neurocytoprotective potential of the novel sodium-calcium ion channel modulator, lifarizine (RS-87476), in two rodent 72 h survival models of forebrain ischaemia.

2 Under fluothane anaesthesia, rats were subjected to 10 min four vessel occlusion and gerbils to either (i) 5 or (ii) 10 min bilateral carotid artery occlusion.

3 Rats were dosed parenterally solely post-ischaemia (reperfusion) in a series of five studies covering a range of intra-arterial/intraperitoneal (i.a./i.p.) combination doses from 2/10, 5/20, 20/100, 50/200 and 100/500 $\mu g\ kg^{-1}$, where the initial loading dose was injected i.a. at 5 min. An i.p. dose was given at 15 min and repeated twice daily. In a sixth study, treatment at 50/200 $\mu g\ kg^{-1}$ was deferred for 1 h.

4 Gerbils were treated (i) 15 min pre-ischaemia with either (a) 250, (b) 500 $\mu g\ kg^{-1}$ i.p., or (c) 5 mg kg^{-1} by gavage (p.o.) for 3 days then at 1 h pre-ischaemia. Animals treated as (ii) received 500 $\mu g\ kg^{-1}$ i.p. 15 min pre-ischaemia. The above doses were repeated twice daily for 3 days post-ischaemia for the respective groups.

5 In rats, the protective effect of lifarizine was regionally and cumulatively assessed in six brain regions (anterior and posterior neocortex, hippocampal CA_1 subfield, thalamus, striatum, cerebellar Purkinje cells-brain stem) at each dose level. Cumulative (total) mean ± s.e.mean neurohistopathological scores (0-4) of 1.16 ± 0.09 ($n=5$), 1.02 ± 0.10 ($n=5$), 0.93 ± 0.06 ($n=6$), 0.79 ± 0.09 ($n=9$) and 0.45 ± 0.16 ($n=7$), respectively, were obtained for the above treatment groups compared to the control (2.01 ± 0.17, $n=16$) group ($P<0.0035$). The score for the 1 h deferred treatment group was also significant at 0.77 ± 0.10, $n=5$ ($P<0.0035$). The normal group without ischaemia showed a score of 0.52 ± 0.09 ($n=6$).

6 In gerbils, (i) percentage delayed neuronal death (DND) of hippocampal pyramidal cells in the CA_1 subfield was prevented at 250 (a) and 500 $\mu g\ kg^{-1}$ i.p. (b) (27.2 ± 14.6, $n=6$ and $26.9 \pm 10.4\%$, $n=10$ respectively, $P<0.02$) compared to controls ($78.3 \pm 8.5\%$, $n=12$) and by 5 mg kg^{-1} p.o. (c) ($2.9 \pm 0.8\%$, $n=11$, $P<0.002$). Mean ± s.e.mean total brain scores (0-4) for each of 4 different features denoting cerebral 'oedema' were lower for normal brains (1.60 ± 0.34, $n=6$) and reduced in animals dosed at 250 (a) (3.00 ± 0.79, $n=6$) and 500 $\mu g\ kg^{-1}$ i.p. (b) (3.75 ± 0.36, $n=10$) compared to controls (6.58 ± 1.00, $n=12$) ($P<0.02-0.03$). There was a linear relationship ($r=0.97$) between the 'oedema' scores and percentage CA_1 DND. Percentage CA_1 DND in response to 10 min ischaemia (ii) was reduced ($53.0 \pm 21.0\%$, $n=6$, $P<0.05$) compared to controls ($100.0 \pm 0.0\%$, $n=7$).

7 The significant neuroprotection shown by lifarizine in rodents substantiates findings in other species. These observations, together with its effect on ion channels and efficacy at extremely low doses offers novelty and suggests a broad spectrum of activity in ischaemia.

Keywords: Lifarizine (RS-87476), forebrain ischaemia; neurocytoprotection in rodents

Introduction

The use of a suitable animal model facilitates our understanding of the pathophysiology and the pharmacological interventions necessary in the treatment of cerebral ischaemia. Rodent models of transient cerebral ischaemia such as four vessel occlusion (4VO) in the rat (Pulsinelli & Brierley, 1979; Alps & Hass, 1987) and bilateral carotid artery occlusion in the gerbil (Kirino, 1982) develop a reproducible and quantifiable morphological lesion in some brain areas (Alps *et al.*, 1988; Ginsberg & Busto, 1989). It is evident that the damage in selectively vulnerable regions worsens with time, indicating that the cascade of biochemical events initiated by ischaemia progresses even after tissue reperfusion (Pulsinelli *et al.*, 1982; Siesjo & Wieloch, 1985). Neurones may appear morphologically normal up to 24 h post-ischaemia only to deteriorate over the second and third day after the ischaemia event (Kirino, 1982).

Lifarizine, RS-87476 (1-[(2-(4-methylphenyl)-5-methyl)-1*H*-imidazol-4-yl-methyl]-4-diphenyl-methyl-piperazine, Figure 1) is a novel ion channel modulator, active at both Na^+ and Ca^{2+} channels (Alps, 1992). It shows features similar to some class III type calcium entry blocking agents (Spedding, 1985) with regard to effects on Ca^{2+} channels and marked inhibition of Ca^{2+} channel activators (Fraser & Spedding, 1991).

The objective of this study was to evaluate the broad neurocytoprotective potential of lifarizine over a wide range of doses administered parenterally during the post-ischaemic reperfusion period in rats and pre-ischaemia via parenteral or the oral routes in gerbils. Animal brains were prepared for histological endpoint examination 72 h post-ischaemia. A preliminary account of the neuroprotective effect of lifarizine on mean brain scores in the rat 4VO model of forebrain ischaemia was presented at the Satellite Symposium to the ISIM 90 Congress in Sweden (Alps *et al.*, 1990).

Methods

Induction of forebrain ischaemia in rats

Transient forebrain ischaemia was induced in male Sprague-Dawley rats (237-334 g) by a two stage surgical procedure,

[1] Author for correspondence.

adapted from Pulsinelli & Brierley (1979). Details of the technique used for stage one have been described elsewhere (Alps & Hass, 1987). It was essential at this stage to ensure complete interruption of the vertebral arterial supply in the alar foramen. Stage two has been modified to improve anaesthetic control over the carotid (ictal) pressor response evoked when the blood supply to the brain is suddenly reduced, and to ensure effective support of respiration if spontaneous breathing ceased. Two overhead operating table lamps, giving an ambient air temperature of about 25–26°C during the entire stage-two surgical procedure were used to maintain whole body temperature, including the head, at 37°C. Core temperature was monitored with a thin glass bulbed clinical thermometer (units were converted from °F to °C).

After stage one the animals were fasted overnight and anaesthetized in a bell jar with 5% fluothane in a 30% oxygen: 70% nitrous oxide gas mixture at a flow rate of 1 l min^{-1}. Once anaesthetized they were transferred to a loosely fitting face mask and the fluothane concentration decreased to 1–1.5%. A bitemporal EEG was recorded from needle electrodes inserted subcutaneously (50 $\mu V\ cm^{-1}$, 30 mm s^{-1}) on an EEG 10 inkspray recorder (Siemens Ltd, Edinburgh, Scotland). A baseline blood glucose estimation was made using a Reflocheck digital reflectance meter (Boehringer Co. [London] Ltd. U.K), and repeated following restoration of EEG activity. Blood pressure and heart rate were recorded continuously with a Siemens 82 inkspray recorder and a 746 pressure transducer via a cannulated tail artery. The common carotid arteries were exposed for temporary occlusion through a ventral midcervical incision and a loose ligature placed round the vessels. The animals were then transferred to a tightly fitting latex face mask, stretched over the face and mouth of the rats, and attached to a small animal ventilator (Bioscience, Kent). Anaesthesia was maintained by attaching the anaesthetic apparatus to the input of the ventilator, thereby ensuring continuous control of both anaesthesia and ventilation. Anaesthesia was controlled throughout the duration of the experimental procedure with vaporizer A (Figure 2) apart from the short period immediately following ligation when vaporizer B was added to defeat rapidly the ictal pressor response.

One minute prior to the induction of transient (10 min) forebrain ischaemia the fluothane was discontinued. Immediately following the occlusion of the common carotid arteries, with atraumatic clips (Acland C-2-V; Weiss, London), both vaporizers set to 5% were used to defeat the ictal pressor response. Once the EEG had become isoelectric vaporizer B was switched off and control reverted to vaporizer A only. The fluothane concentration was adjusted to maintain a blood pressure of 40–50 mmHg until 9 min ischaemia when it was again discontinued. Providing the blood pressure at release was 50–55 mmHg spontaneous and sustained respiration could be achieved within 2–3 min and EEG activity would return within 1000–1200 s.

Prior to surgical repair of the wounds a post-ischaemia blood glucose estimation was made. The rats were allowed to recover for 72 h, singly housed with free access to food and water. Observations of overt behaviour were made throughout the survival period and animals which developed seizures or inability to feed were to be removed from the study.

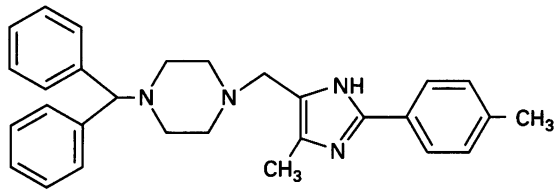

Figure 1 Structure of lifarizine (RS-87476).

Induction of forebrain ischaemia in gerbils

A detailed description of the methodology used to induce forebrain ischaemia in gerbils has been presented previously (Alps *et al.*, 1988). Briefly, animals of either sex, weighing 50–80 g, were anaesthetized with fluothane in a 30% oxygen: 70% nitrous oxide gas mixture and subjected either to (i) 5 min, or (ii) 10 min, bilateral carotid artery occlusion with a 72 h post-ischaemia survival period. Maintenance of whole body temperature was ensured as with the rats.

Drug solutions and dosing schedules

Sterile solutions of lifarizine (expressed as base substance) were prepared initially in a 500 μl volume of ethanol and diluted to volume with saline.

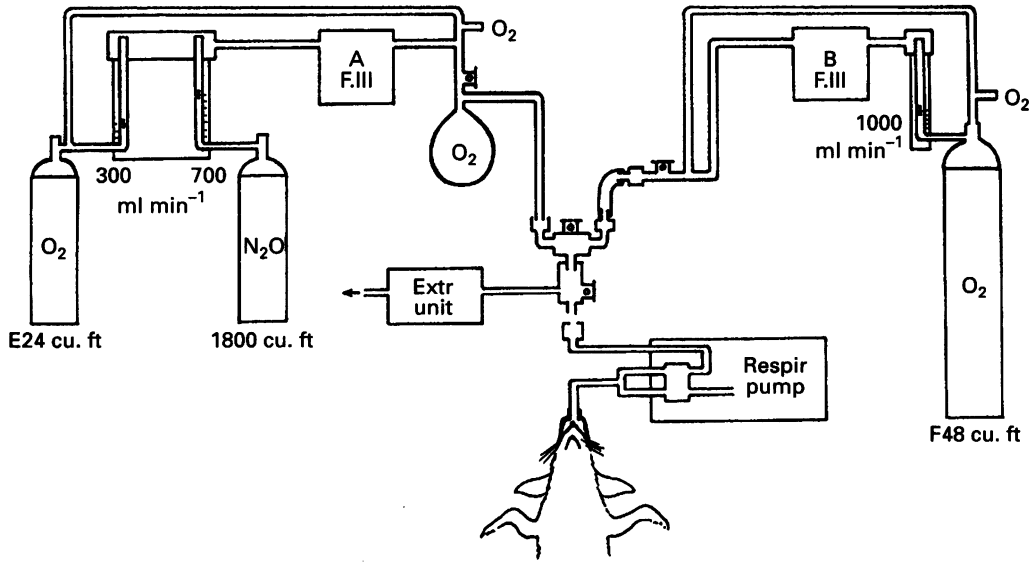

Figure 2 Gaseous anaesthetic apparatus, with two MK III fluotec vaporizers (A,B) used to maintain general anaesthesia and control blood pressure, to inhibit the carotid (ictal) pressor response following occlusion of the common carotid arteries. Excess anaesthetic gas is removed by the extractor (Extr) unit. Respiratory support is given by the respiratory (Respir) pump.

For rats the concentration of stock solution was calculated to give 0.05 ml i.a. and 0.2 ml i.p. 100 g^{-1} bodyweight respectively. Group A received the saline vehicle via the same routes and in similar volumes to animals receiving lifarizine. Five different i.a./i.p. dose ($\mu g\ kg^{-1}$) combinations of lifarizine were administered as follows: groups B = 2/10; C = 5/20; D = 20/100; E = 50/200; F = 100/500. The initial i.a. loading dose was administered after 5 min reperfusion with the accompanying i.p. dose 10 min later. A sixth group, G, was given 50/200 but the initial i.a. loading dose was deferred for 1 h, with the accompanying i.p. dose 10 min after that. Thereafter, repeat maintenance injections of the i.p. dose used for each group were given twice daily during the 72 h survival period. Brains from normal animals (group H) were included for comparison.

For gerbils, the concentration of the lifarizine stock solution was 100 $\mu g\ ml^{-1}$. In animals subjected to (i) 5 min ischaemia, three different treatment regimens were followed: (a) 250 or (b) 500 $\mu g\ kg^{-1}$ i.p. of sterile solutions of lifarizine were injected 15 min before carotid artery occlusion, or (c) 5 mg kg^{-1} was given p.o. twice daily for 3 days, then at 1 h pre-ischaemia on day 4. Animals subjected to (ii) 10 min ischaemia received lifarizine, 500 $\mu g\ kg^{-1}$ i.p., at 15 min pre-ischaemia. These doses were repeated twice daily in the respective groups during the 72 h post-ischaemia survival periods. Since the data for saline-treated controls and normal gerbils is the same as that reported elsewhere (Alps *et al.*, 1988) and was produced at the same time as lifarizine was evaluated, it is referred to directly in this paper for purposes of comparison and statistical analysis.

Preparation of tissues for histopathological examination

Following 72 h reperfusion each rat or gerbil was anaesthetized with pentobarbitone sodium (120 mg kg^{-1}, i.p.) and thoracotomized. The cerebral circulation was flushed with heparinised saline through an intra-cardiac injection (left ventricle), and perfuse-fixed with 10% formalin. The whole heads were then removed and stored overnight in 10% formalin at 4°C. The brains were removed and stored in fresh fixative for 4–5 days before trimming for histological examination.

In rats, coronal sections were taken at three levels (1) divergence of the optic chiasma, (2) mid-point of the infundibulum, (3) cerebellar-brain stem. The slices were embedded in separate blocks of paraffin wax and sectioned at 7 μM. Alternate sections were stained with haematoxylin/eosin and cresyl fast violet.

In gerbils, coronal slices of brains were examined at selected co-ordinates according to Kirino (1982). Artefactual changes were allowed for by fixing and processing parallel perfusion controls with the ischaemic animal brains.

Assessment of neuropathological damage and analysis of data

Neuropathological damage was assessed in terms of ischaemic cell change (ICC) as described by Brown & Brierley (1968). All areas were examined at × 100 and × 400 magnification and the sections from the treated groups were graded blind along with normal and drug vehicle-treated control brains to decrease bias.

In the rat the brain areas examined were (i) layers I–III and IV–VI of the anterior and posterior neocortex, (ii) hippocampal CA_1 subfield, (iii) thalamus, (iv) striatum, (v) cerebellar Purkinje cells - brain stem. Each area was graded for % ICC on a 0–4 basis according to the procedure previously determined for this 4VO model (Alps & Hass, 1987) as modified from that described originally by Pulsinelli & Brierley (1979). Thus: 0 = Normal; 0–1 = 0–10%; 1–2 = 10–25%; 2–3 = 25–50%; 3–4 = > 50%.

For each animal the hippocampal and striatal cortical assessments were given overall worst and best scores and from these an average value was calculated. Scores for hippocampal CA_1 subfield, striatum, and thalamus were assessed separately

Table 1 Measurements of the onset time and duration of the isoelectric electroencephalogram (EEG), blood pressure and heart rate in rats subjected to 10 min forebrain ischaemia

Treatment *Dose ia/ip* ($\mu g\ kg^{-1}$)	*Group size* *n* =	*Isoelectric EEG* *Onset* (s)	*Duration* (s)		*Blood pressure* {Systolic (S), Diastolic (D), mmHg} and *Heart rate* (HR), beats min^{-1}] *Starting level*	*Pre carotid occlusion*	*Peak pressor*	*Onset of EEG isoelectricity*	*Return of EEG*	*Final (end stage 2)*
A	16	71.9 ± 14.5	1105.1 ± 47.7	S	73.1 ± 2.3	84.8 ± 1.9	115.6 ± 4.0	85.9 ± 7.3	101.4 ± 4.9	108.3 ± 2.8
Vehicle				D	62.3 ± 2.6	74.0 ± 1.8	105.4 ± 3.1	75.9 ± 7.1	90.1 ± 5.2	99.9 ± 3.0
Control				HR	329.0 ± 8.0	342.0 ± 8.1	325.0 ± 12.4	294.0 ± 6.4	342.0 ± 11.1	418.0 ± 15.9
B	5	73.8 ± 16.8	1056.2 ± 39.3	S	78.0 ± 3.0	84.0 ± 7.5	123.0 ± 9.3	79.0 ± 15.7	118.0 ± 8.2	116.0 ± 8.7
2\10				D	73.0 ± 3.0	79.0 ± 7.5	118.0 ± 9.3	74.0 ± 15.7	111.0 ± 7.1	110.0 ± 8.5
				HR	334.0 ± 11.7	316.0 ± 14.7	318.0 ± 12.0	302 ± 19.6	344.0 ± 11.7	398.0 ± 13.6
C	5	50.0 ± 15.2	1058.0 ± 38.4	S	62.0 ± 2.0	67.0 ± 2.5	105.0 ± 7.1	81.0 ± 5.1	96.0 ± 4.3	88.0 ± 2.6
5\20				D	57.0 ± 2.0	62.0 ± 2.5	100.0 ± 7.1	76.0 ± 5.1	90.0 ± 5.0	83.0 ± 2.6
				HR	356.0 ± 9.8	348.0 ± 8.0	360.0 ± 6.3	336.0 ± 9.8	410.0 ± 4.5	402 ± 11.1
D	6	71.5 ± 21.6	1120.0 ± 69.9	S	70.0 ± 2.2	75.0 ± 2.2	113.3 ± 6.0	83.3 ± 13.3	118.3 ± 5.8	105.0 ± 3.7
20\100				D	65.0 ± 2.2	70.0 ± 2.2	108.3 ± 6.0	78.3 ± 13.3	113.3 ± 5.8	100.0 ± 3.7
				HR	326.0 ± 6.7	330.0 ± 8.6	330.0 ± 11.3	317.0 ± 9.5	413.3 ± 17.6	398.0 ± 12.2
E	9	44.6 ± 14.3	1081.0 ± 35.7	S	63.1 ± 2.9	69.4 ± 4.5	100.6 ± 4.9	80.6 ± 9.1	96.1 ± 5.1	97.2 ± 6.2
50\200				D	58.3 ± 3.0	64.4 ± 4.5	95.6 ± 4.9	75.6 ± 9.1	91.1 ± 5.1	92.2 ± 6.2
				HR	341.0 ± 7.2	344.0 ± 5.6	338.0 ± 5.2	330.0 ± 6.7	389.0 ± 13.8	387.0 ± 17.2
F	7	20.1 ± 4.2	1206.3 ± 78.2	S	76.4 ± 1.8	77.1 ± 2.9	111.4 ± 3.4	100.0 ± 5.3	114.3 ± 2.8	122.9 ± 5.7
100\500				D	67.9 ± 2.4	68.6 ± 3.0	99.3 ± 3.4	90.0 ± 5.6	100.7 ± 4.0	108.6 ± 4.8
				HR	338.0 ± 8.2	319.0 ± 8.8	326.0 ± 9.2	316.0 ± 9.3	336.0 ± 14.6	383.0 ± 20.2
G	5	60.0 ± 17.2	896.0 ± 71.5	S	77.0 ± 3.0	77.0 ± 5.1	109.0 ± 4.8	71.0 ± 16.1	107.0 ± 3.4	107.0 ± 6.0
50\200				D	72.0 ± 3.0	72.0 ± 5.1	104.0 ± 4.8	66.0 ± 16.1	102.0 ± 3.4	102.0 ± 6.0
1 h deferred				HR	390.0 ± 20.5	372.0 ± 13.6	360.0 ± 18.9	344.0 ± 23.2	400.0 ± 31.0	390.0 ± 22.0

Animals in group A treated with saline i.a./i.p. Values are mean ± s.e.mean. Isoelectric times are in s. Blood pressure (systolic/diastolic) is in mmHg and heart rate (HR) beats min^{-1}.

for each hemisphere and the mean derived. A single assessment of ICC to the cerebellar Purkinje cells was given. A whole brain score for each animal could therefore be calculated from the regional values. These individual values were meaned to give a group mean ± s.e.mean. The whole brain scores were calculated with and without the CA_1 subfield component. Students t test for unpaired samples, one-tailed with Bonferroni's modification for multiple comparisons ($\times 7$) against a single control group was used. The criterion of significance was taken as $P<0.05$.

In the gerbil for animals evaluated in the (i) 5 min ischaemia group and dosed at 250 or 500 $\mu g\ kg^{-1}$ i.p., the extent of neuronal damage was examined in two ways, (a) percentage values were determined for delayed neuronal death (DND) of CA_1 cells in five areas covering the hippocampus in each hemisphere so that regional involvement and symmetry of the lesion could be examined, (b) from the 10 subfields individual animal mean values for percentage DND were calculated and used to determine group values. A repeated measures analysis of variance was performed with grouping factor treatment and the repeated measures factors hemisphere and position (Alps *et al.*, 1988). The differences between the five positions were broken down into orthogonal polynomials. Initially, the hypothesis that the treatment did not affect the differences between the ten subfields was tested by F-tests. These were not statistically significant and therefore the active treatments were compared to control using animal means by t tests (Snedecor & Cochran, 1976) with standard errors derived from the analysis of variance. These were one-tailed since, *a priori*, it was believed that the treatments would, if anything, reduce percentage CA_1 neuronal death. A Bonferroni adjustment was made for multiple group comparisons and the criterion of significance was taken where $P<0.05$.

In addition, four features appearing in each gerbil brain section which suggested oedematous changes and, or, more general damage to the neuropil and structures outside the

Table 2 The effect of lifarizine treatment on whole body (core) temperature in rats subjected to 10 min forebrain ischaemia

Treatment dose i.a./i.p. ($\mu g\ kg^{-1}$)		*Rectal temperature* (°C) *Pre-ischaemia*	*During ischaemia*	*Post-ischaemia*
A Saline Controls	($n=16$)	37.0 ± 0.09	36.9 ± 0.07	37.0 ± 0.05
B 2/10	($n=5$)	36.9 ± 0.15	36.9 ± 0.14	36.9 ± 0.13
C 5/20	($n=5$)	36.9 ± 0.15	36.9 ± 0.08	36.8 ± 0.12
D 20/100	($n=6$)	37.1 ± 0.18	36.9 ± 0.15	36.9 ± 0.19
E 50/200	($n=9$)	36.9 ± 0.19	37.2 ± 0.16	37.0 ± 0.15
F 100/500	($n=7$)	36.9 ± 0.14	36.9 ± 0.15	36.9 ± 0.13
G 50/200 1 h deferred	($n=5$)	37.0 ± 0.15	37.0 ± 0.16	37.1 ± 0.19

Values are mean ± s.e.mean. Statistical significance was assessed with Student's two tailed t test with adjustments for 7 comparator groups against the control group using Bonferroni's method. No lifarizine group reading was significantly different from comparative control group values.

Table 3 Protective effects of lifarizine against regional ischaemic cell changes induced in the rat brain following four vessel occlusion

Treatment dose (i.a./i.p. $\mu g\ kg^{-1}$)	*Hippocampal CA1 cells*	*Hippocampal cortex*	*Striatum*	*Striatal cortex*	*Thalamus*	*Cerebellum brain stem*
A Vehicle control $n=16$	3.52 ± 0.21	1.66 ± 0.27	1.95 ± 0.23	1.49 ± 0.25	2.05 ± 0.22	1.41 ± 0.19
B 2\10 $n=5$	1.53 ± 0.20 **	0.85 ± 0.05 *	1.03 ± 0.20 *	0.85 ± 0.05 NS	1.45 ± 0.05 NS	1.20 ± 0.20 NS
C 5\20 $n=5$	2.05 ± 0.43 *	0.69 ± 0.15 *	0.95 ± 0.12 *	0.45 ± 0.15 *	1.35 ± 0.10 *	0.70 ± 0.12 *
D 20\100 $n=6$	2.44 ± 0.63 NS	0.42 ± 0.10 **	0.54 ± 0.10 **	0.21 ± 0.07 **	0.96 ± 0.20 *	0.92 ± 0.24 NS
E 50\200 $n=9$	1.00 ± 0.19 **	0.44 ± 0.12 **	0.72 ± 0.17 **	0.85 ± 0.18 NS	0.97 ± 0.18 **	0.78 ± 0.17 NS
F 100\500 $n=7$	1.14 ± 0.53 **	0.14 ± 0.09 **	0.39 ± 0.27 **	0.18 ± 0.09 **	0.39 ± 0.27 **	0.43 ± 0.17 **
G 1 h deferred 50\200 $n=5$	2.05 ± 0.58 NS	0.25 ± 0.11 **	0.35 ± 0.06 **	0.20 ± 0.05 **	1.00 ± 0.08 **	0.75 ± 0.27 NS
H Normals $n=6$	0.33 ± 0.11 **	0.31 ± 0.06 **	0.79 ± 0.19 **	0.36 ± 0.12 **	0.79 ± 0.19 **	0.50 ± 0.18 *

Values are mean ± s.e.mean, calculated from individual animal scores. Statistical analysis was performed by Student's single tailed t test with adjustments for 7 comparator groups against the control group using Bonferroni's method.
*$P<0.035$, **$P<0.0035$.

hippocampus were quantified by scoring each feature on a 0–4 basis (Alps, 1992). The features examined were: (i) splitting of the neuropil in the hippocampal fissure, (ii) disruption of the neuropil around the dentate, (iii) dilatation of the lateral ventricles, and (iv) presence of perinuclear 'halos' extending progressively throughout the whole brain slice from the midline and paramedian areas. The latter was taken as evidence of previous intracellular swelling. The overall relationship between the group mean scores of general brain involvement and the group percentage values for DND were examined by applying the Pearson correlation coefficient.

Results

Effects of lifarizine in rats

Mortality in rats subjected to four vessel occlusion was low in this study with four deaths: two animals (1 control, 1 group C) died overnight from unknown causes: two animals (group F) were eliminated from the study prior to dosing with lifarizine: one died following seizures and a second was terminated due to a locomotor deficit following stage-one surgery. All survivors remained free from seizures and locomotor deficit. The treated animals showed a more normal overt behavioural pattern than the vehicle controls. From the estimations made before and after the procedures there was no significant change in whole blood glucose in any treatment group or the controls in this study.

Data for onset and duration of the isoelectricity for each group are shown in Table 1. The groups had a mean EEG isoelectric duration of about 1000–1200 s, although group G was slightly less.

Blood pressure and heart rate data for each group are also shown in Table 1 at equivalent time points and doses used in this study. There appeared to be no drug-related changes in the haemodynamic parameters measured in this study. Core body temperature (°C) was not affected by, or during, the surgical-ischaemic procedure itself or by administration of lifarizine in any group of animals (Table 2).

Data for the regional brain scores (mean ± s.e.mean) for ICC for each treatment group compared to vehicle control and normal animals are shown in Table 3. Whole brain scores (mean total scores, with and without the CA_1 component included) are shown in Figure 3. The regional (except for the hippocampal CA_1 neurones) and whole brain scores showed a dose-related protective effect.

Apart from group D, all other 5 min reperfusion-treated groups showed significant protection of the CA_1 neurones ($P<0.0035-0.035$). All treatment groups showed significant protection of the hippocampal cortex and striatum ($P<0.0035-0.035$). Groups B and E showed a non significant reduction in the damage to the striatal cortex. Significant protection to the CA_1 neurones was not found in the 1 h deferred treatment group G.

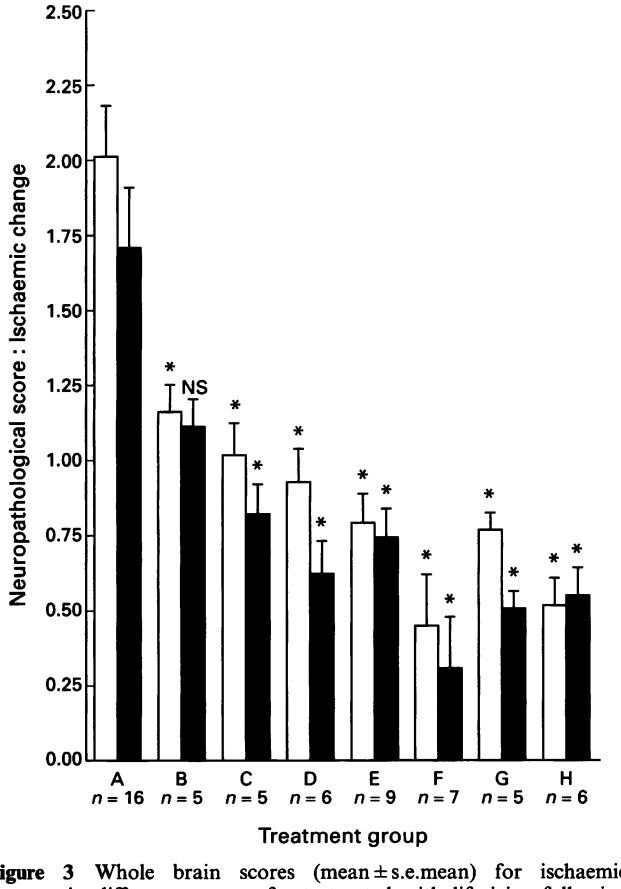

Figure 3 Whole brain scores (mean ± s.e.mean) for ischaemic damage in different groups of rats treated with lifarizine following 4 vessel occlusion, in comparison with untreated controls (A) and normal brains (H). The mean scores were calculated with (open columns), and without (solid columns) the CA_1 component value to demonstrate that protection was achieved in structures outwith the hippocampus. Treatment with lifarizine consisted of a series of i.a./i.p. doses ($\mu g\,kg^{-1}$) given 5 min (i.a.) and at 15 min (i.p.) post-reperfusion as follows: group B = 2/10, C = 5/20, D = 20/100, E = 50/200, F = 100/500. In group G, dosing (50/200) was deferred until 1 h post reperfusion. Statistical analysis was performed with Student's single tailed *t* test with adjustments for 7 comparator groups against the control group using Bonferonni's method. $*P<0.0035$.

Effects of lifarizine in gerbils

In the non-drug treated gerbils subjected to (i) 5 min bilateral carotid artery ischaemia, the extent of total DND in the whole hippocampal CA_1 subfield was 78.3% (Table 4). Abnormal neurones appeared in non-ischaemic gerbil brains to the extent of 4.5% (Table 4); these changes can be ascribed to fixation artefacts.

Lifarizine (250 or 500 $\mu g\,kg^{-1}$, i.p. 15 min prior to the ischaemic insult and then twice daily for the three day recovery period) markedly reduced hippocampal CA_1 DND in a bilaterally symetrical manner throughout the hemispheres of the gerbil brain ($P<0.003$, Table 4, Figure 4). Both of these doses of lifarizine also significantly prevented ($P<0.02-0.03$) the oedematous changes which took place in the gerbil brain after ischaemia. There was a linear relationship ($y=14.7\ x-20.6$, $r=0.97$) between the mean of the total 'oedema' scores and extent of percentage DND for the CA_1 subfield. The 5 mg kg^{-1} p.o. dose regimen (ischaemic insult [i]) was also significantly neuroprotective ($P<0.002$, Table 4).

In the group of 10 untreated gerbils subjected to 10 min carotid artery occlusion (ischaemic insult [ii]), one animal died and two were not considered valid to include for technical reasons (unilateral hemispheric damage only). Damage was assessed at 100.0 ± 0.0% for the 7 remaining animals. In the lifarizine group of 10 animals, two died and two were excluded for the same technical reasons as for the controls. In this group damage was assessed at 53.0 ± 21.0% for the 6 remaining animals ($P<0.05$ compared to controls). Lifarizine was therefore protective against delayed neuronal cell death in the CA_1 region following ischaemia lasting for 5 and 10 min.

Lifarizine had no obvious effects on the behaviour of the gerbils in these experiments, except that the untreated animals were subdued and the treated animals were alert and groomed themselves normally; these beneficial effects are presumed due to the neuroprotective effects of lifarizine, rather than any specific behavioural effects.

Discussion

In the rat model of forebrain ischaemia it was evident that 10 min bilateral arterial occlusion *per se* did not imply an

Table 4 The effect of lifarizine treatment on gross cerebral oedema damage and the percentage delayed neuronal death in the hippocampal CA1 subfield of the gerbil brain

Group treatment	*Number of animals*	*% abnormal neurone count*	*Total oedema score*
Normals	10	4.5 ± 1.1**	1.60 ± 0.34**
Saline controls	12	78.3 ± 8.5	6.58 ± 1.00
Lifarizine (250 μg kg^{-1}, i.p)	6	27.2 ± 14.6**	3.00 ± 0.79*
Lifarizine (500 μg kg^{-1}, i.p)	10	26.9 ± 10.4**	3.75 ± 0.36*
Lifarizine (5 mg kg^{-1}, po)	11	2.9 ± 0.8***	Not assessed

Statistical significance was assessed from modified t tests with Bonferroni adjustment for multiple comparisons. Significance where $P < 0.05$. *$P < 0.02-0.03$, **$P < 0.015-0.02$, ***$P < 0.0015-0.002$. Data are mean ± s.e.mean.

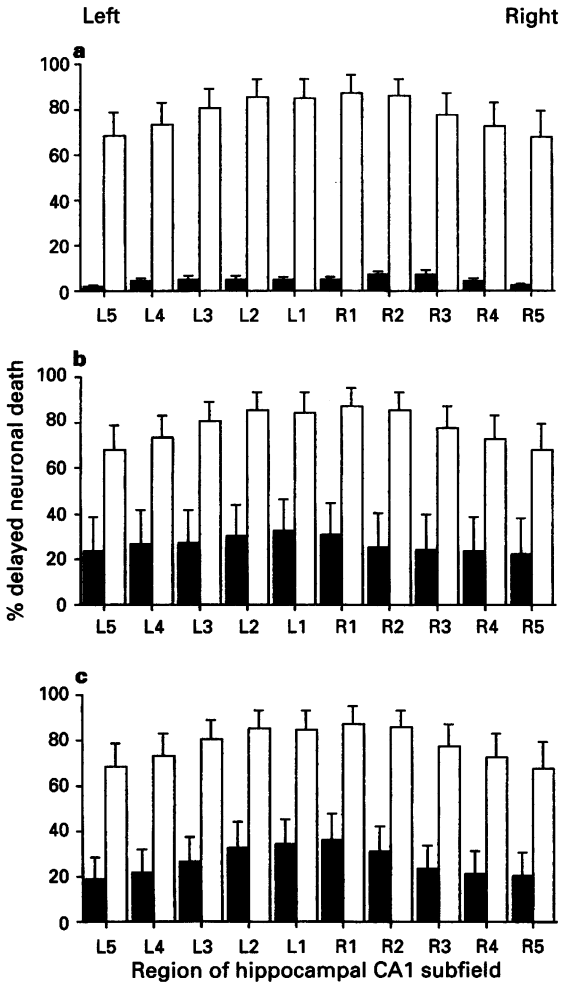

Figure 4 Protective effect of lifarizine administered pre-ischaemia i.p., with repeated i.p. maintenance dosing twice daily, against delayed neuronal death (DND) in the hippocampal CA_1 subfield determined histologically for 5 regions throughout each hemisphere in the brain of gerbils subjected to 5 min bilateral carotid artery occlusion with 72 h survival. The area examined was cut coronally approximately 1.4 mm posterior to the bregma, extending from the paramedian (region 1) to the junction with the CA_2 neurones (region 5). Histograms (open columns) for each region in the three panels (a,b,c) show % distribution DND (mean ± s.e.mean) in the same saline-treated gerbils ($n = 12$). These values are compared to those (solid columns) for neurones exhibiting changes attributed to fixation artefacts in normal animals (panel a, $n = 10$), a group treated with 250 μg kg^{-1}, i.p. (panel b), and a group treated with 500 μg kg^{-1}, i.p. (panel c). Statistical analysis by modified t tests with Bonferroni modification for multiple comparisons ($P < 0.05$) gave significance at each region for both regimens in both hemispheres ($P < 0.002-0.003$).

equivalent period of effective ischaemia, and within groups control was exerted over the peripheral blood pressure and ventilation to achieve as near as possible an optimum isoelectric period of 1000–1200 s (Alps *et al.*, 1990) to ensure reproducible neuronal damage. Careful control of pulmonary ventilation and anaesthesia was essential to combat the potentially damaging effects of anaesthetic-induced hypotensive damage to the cerebellar Purkinje cells. Thus, in this study no obvious pulmonary complications were observed and mortality was very low. Neuronal sensitivity to ischaemia revealed the following order of selective vulnerability: hippocampal CA_1 > striatum = thalamus > cerebellar Purkinje cells > hippocampal cortex > striatal cortex. This order was similar but not identical to that reported elsewhere (Smith *et al.*, 1984; Alps & Hass, 1987). A remarkable degree of dose-related protection was observed for lifarizine in six different rat brain areas over a very low dose-range, the damage being limited to about 50% of that shown in ischaemic controls by 2 μg kg^{-1}, i.a. with i.p. maintenance at 10 μg kg^{-1}. Moreover, this protection in the rat was achieved without occurring any change in the cardiovascular parameters measured or drug-related behavioural changes. The animals recovered quickly from the surgical procedure and clinically appeared very healthy and, apart from one animal following stage-one surgery, seizures did not occur.

Lifarizine was found to be highly effective in protecting the pyramidal cells in the CA_1 region of the gerbil hippocampus by both the i.p. and p.o. routes. Comparative data previously reported for nimodipine, flunarizine, lidoflazine and nicardipine (Alps *et al.*, 1988) showed that lifarizine was much more protective in this model than nicardipine and flunarizine; lidoflazine was hardly active and nimodipine was inactive. A recent study (Brown *et al.*, 1993) in anaesthetized gerbils demonstrated that the neuroprotective doses of lifarizine used in the present study also protected (9.2% decrease at 250 μg kg^{-1} and no decrease at 500 μg kg^{-1} i.p., $P < 0.01$) against dopamine depletion compared to control animals (62.7% decrease) under conditions of 3 h unilateral ligation of the right common carotid artery.

The CA_1 neurones have L-type Ca^{2+} channels which show increased binding by [^{3}H]-PN200-110 in ischaemia (Magnoni *et al.*, 1988) and are especially vulnerable to ischaemic damage in animal models (Kirino, 1982; Suzuki *et al.*, 1983a,b; Alps *et al.*, 1988; Spedding *et al.*, 1989), and in man (Zola-Morgan *et al.*, 1986), where damage of these cells may be excessively stimulated during the ischaemic reperfusion period via the Schaeffer collaterals which release excitatory amino acids (Suzuki *et al.*, 1983a,b). However, lifarizine does not act as an excitatory amino acid antagonist and does not modify transmission via the Schaeffer collaterals under non-ischaemic conditions but does decrease calcium-dependent glutamate release from rat cerebrocortical synaptosomes and inhibits rises in intracellular [Ca^{2+}] in rat cultured cortical neurones (unpublished observations). It is known that lipid metabolite products of ischaemia can behave like the synthetic activators of Ca^{2+} channels (Bay K8644 and CGP 28392), and are

claimed to slow the transition of the Ca^{2+} channel from the active to the closed state (see Sanguinetti *et al.*, 1986; Kass, 1987). Since it is possible that such agents may have a role to play in the pathogenesis of cerebral ischaemia (Patmore *et al.*, 1989), it is important to consider that, in addition to its blockade of L-type Ca^{2+} channels, the marked inhibition of Ca^{2+} channel activators by lifarizine (Fraser & Spedding, 1991) may constitute an added neuroprotective mechanism.

Cerebral blood flow was not measured either locally or globally during the period of ischaemia in the rat model during this study. However, in other studies to date (unpublished observations) using an *in vivo* autoradiographic technique (^{14}C -iodoantipyrine tracer) the effect after 10 min of a loading (neuroprotective but non-hypotensive) dose of 100 $\mu g\ kg^{-1}$ given either i.a. or i.v. on cerebral blood flow has been examined in both normal anaesthetized ($n=5$ for 35 brain areas) and conscious ($n=6$ for 32 brain areas) rats. There were no significant *net* vasodilator or constrictor effects on blood flow in any area. The areas included those that would be regarded as selectively vulnerable in the ischaemic brain. It has also been confirmed that cerebral blood flow is virtually eliminated in these areas 6 min into the ischaemic phase of non drug-treated animals. In the present study, lifarizine was administered early during reperfusion but whilst the EEG was still firmly isoelectric. It seems unlikely that lifarizine had any cerebrovascular dilator effect at this time since it could be expected that if cerebral blood flow had been improved, the isoelectric EEG period would have been shortened and this was not observed. Equally well, there is no reason to believe that the process of recovery was adversely affected. It has also now been found that whereas an i.v. dose of 1 mg kg^{-1} will lower systemic blood pressure and decrease cerebral blood flow in anaesthetized rats (and in this situation neuroprotection is diminished), this same dose is without effect on blood pressure in conscious animals.

Problems of hypoperfusion including interstitial (neuropil) oedema and vasospasm due to damaged vascular endothelium and calcium overload have been shown to exacerbate neuronal damage (Kazda & Mayer, 1985). The exact mechanism of neuroprotection on the CA_1 pyramidal cells has not been fully defined, but protection in the gerbil brain was not confined to the hippocampus alone in that the overall pattern of oedematous changes in the brain was reduced by lifarizine, indicating a more general protective effect on cellular ionic homeostasis following the ischaemic insult. The linear relationship ($r=0.97$) between the 'oedema' scores and percentage DND of the CA_1 cells was similar to that ($r=0.96$) previously reported in the gerbil for studies on nicardipine, nimodipine, flunarizine and lidoflazine (Alps, 1992). Studies with a cat model of middle cerebral arterial occlusion employing a combination of magnetic resonance imaging and spectroscopy, with tissue oedema being observed as areas of signal hyperintensity on diffusion-and T2 weighed spin-echo images, for up to 12 h post-ischaemia (Kucharczyk *et al.*, 1991) has supported an anti-oedema effect for lifarizine and substantiated its neuroprotective properties. In the cat, lifarizine administered solely post-ischaemia (2-50 $\mu g\ kg^{-1}$, i.v. loading doses with i.v. maintenance infusion from 0.7-17.5 $\mu g\ kg^{-1}$ respectively) was shown to be effective in reducing severe oedematous changes over a 12 h period and markedly inhibiting the size of resulting infarcts by at least 70%. It is, however, important to note that false negative results have been observed by other investigators Rataud *et al.* (1994) using lifarizine (100 mg kg^{-1}, i.p.) in a rat middle cerebral artery occlusion model of focal ischaemia. They selected the dose of lifarizine on the basis of information contained in the paper by Kucharczyk *et al.* (1991), where unfortunately the doses used in the cat were incorrectly stated in mg kg^{-1} rather than $\mu g\ kg^{-1}$. It has since been confirmed that an i.a./i.p. dose regime of 100/500 $\mu g\ kg^{-1}$ which significantly protects the various brain areas in the rat 4VO model also protects these same areas in a modified rat 12 min 2VO model (McBean *et al.*, 1995a), and an equivalent i.v./i.p. dose regime significantly protects the cortex in the rat rose Bengal model of focal cerebral ischaemia (McBean *et al.*, 1995b). In a recent report by Squire *et al.* (1994) describing the results of a double blind, randomized, parallel group, pilot study in human stroke, lifarizine was well tolerated in elderly patients. Both mortality and functional assessment data showed favourable trends with active treatment but it remains to be seen whether lifarizine will be proved efficacious in such human patients.

The importance of changes in the brain temperature influencing the outcome of ischaemic injury has been reported (Busto *et al.*, 1987; Ginsberg & Busto, 1989; Minamisawa *et al.*, 1990; Welsh *et al.*, 1990; Welsh & Harris, 1991). The neurocytoprotective effect of relative hypothermia has been stressed and adds a variable which must be controlled. During method development and in this study, care was taken to ensure that all animals remained normothermic (stable core temperature 36.8-37.2°C) during surgery and throughout the post-operative recovery period. Skull temperature was not measured in the present study, since placement of recording thermistor probes would have interferred with the delicate and very sensitive recording of the EEG. It is interesting that Ginsberg & Busto (1989) showed in global ischaemia that moderate-marked injury to the CA_1 hippocampal pyramidal neurones occurred in 100% of brains held at 36°C during 20 min of ischaemia and that the percentage injury fell to 20% at 34°C and 0% at 33-30°C. Similar reductions in dorsolateral striatal damage occurred as brain temperature dropped. Welsh & Harris (1991) observed a lack of neuroprotection by acute post-ischaemic hypothermia lasting 1-2 h, and made the comments that the effect on hippocampal injury of pharmacological agents administered during the early phase of recirculation may not be greatly perturbed by transient hypothermia. In the present study all animal experimentation was carried out under the same environment conditions and parallel drug vehicle-treated controls showed good reproducibility of damage when compared to the test groups. In other studies in our laboratory, lifarizine at 10 mg kg^{-1}, i.p. has been shown to have no effect on core temperature in mice (ambient temperature 21-23°C), compared to dose-related hypothermia induced by quinpirole at 0.03-10 mg kg^{-1}, i.p. (unpublished observations). It is therefore difficult to believe that lifarizine would have decreased brain temperature to a hypothermic protective level at the highest dose level (100/500 $\mu g\ kg^{-1}$, i.a./i.p.) employed in the present rat study.

Mechanistically, lifarizine is not a classical calcium antagonist (Alps, 1992) and did not show cardiovascular effects in rats at the neuroprotective doses reported in this paper. It is unlikely that a central neuroprotective agent that acts at a single site will be effective because of the multiple mechanisms involved in the pathology. The effect of lifarizine on at least two types of ion channels involving Na^+ and Ca^{2+}, together with its additional inhibitory effect on Ca^{2+} channel activators and marked effectiveness *in vivo* at extremely low doses, offers novelty and robustness to its mode of action and suggests a unique spectrum of action on a broad variety of neuronal cell types.

In conclusion, within the standardized experimental conditions described for the rat and gerbil models of forebrain ischaemia in this study, lifarizine showed a significant degree of dose-related neuroprotection at very low doses in the brain areas observed. Its potential for treating cerebral ischaemia can further be appreciated by the general benefit achieved when the treatment intervention was deferred for 1 h post reperfusion.

The authors are grateful to Dr J.G. McCormack for the information regarding the effects of lifarizine on intracellular Ca^{2+} and glutamate exocytosis, Dr R. Sheridan and Mr D. Bonhaus for the information on its lack of effect on neuroexcitation, and Dr W. Redfern for observations on lifarizine's lack of effect on body temperature in mice. Information on cerebral blood flow was provided by Dr D.E. McBean.

References

ALPS, B.J. (1992). Drugs acting on calcium channels: potential treatment for ischaemic stroke. *Br. J. Clin. Pharmacol.*, **34**, 199–206.

ALPS, B.J., CALDER, C., HASS, W.K. & WILSON, A.D. (1988). Comparative protective effects of nicardipine, flunarizine, lidoflazine and nimodipine against ischaemic injury in the hippocampus of the Mongolian gerbil. *Br. J. Pharmacol.*, **93**, 877–883.

ALPS, B.J., CALDER, C., WILSON, A.D. & PASCAL, J.-C. (1990). Cerebral protection with a novel calcium blocker in rats. Satellite Symposium to the ISIM 90 Congress '*Acute Care of Stroke*', Djuronaset, Sweden, June 14–17.

ALPS, B.J. & HASS, W.K. (1987). The potential beneficial effect of nicardipine in a rat model of transient forebrain ischaemia. *Neurology*, **37**, 809–814.

BROWN, A. & BRIERLEY, J.B. (1968). The nature, distribution and earliest stages of anoxic-ischemic nerve cell damage in the rat brain as defined by the optical microscope. *Br. J. Exp. Pathol.*, **49**, 87–106.

BROWN, C.M., CALDER, C., ALPS, B.J. & SPEDDING, M. (1993). The effect of lifarizine (RS-87476), a novel sodium and calcium channel modulator, on ischaemic dopamine depletion in the corpus striatum of the gerbil. *Br. J. Pharmacol.*, **109**, 175–177.

BUSTO, R., DIETRICH, W.D., GLOBUS, M.Y.-T., VALDES, I., SCHEINBERG, P. & GINSBERG, M.D. (1987). Small differences in intraischemic brain temperature critically determine the extent of ischemic neuronal injury. *J. Cereb. Blood Flow Metab.*, **7**, 729–738.

FRASER, S. & SPEDDING, M. (1991). RS-87476, a novel neuroprotective agent, antagonises the effects of calcium channel activators in guinea-pig taenia. *Br. J. Pharmacol.*, **104**, P60.

GINSBERG, M.D. & BUSTO, R. (1989). Rodent models of cerebral ischemia. *Stroke*, **20**, 1627–1642.

KASS, R.S. (1987). Voltage-dependent modulation of cardiac calcium channel current by optical isomers of Bay K8644: implications for channel gating. *Circ. Res.*, **61**, (Suppl. 1). 1–5.

KAZDA, S. & MAYER, D. (1985). Postischemic impaired reperfusion and tissue damage: consequences of a calcium-dependent vasospasm? In *Calcium Entry Blockers and Tissue Protection*. ed. Godfraind, T., Vanhoutte, P.M., Govoni, S. & Paoletti, R. pp 129–138. New York: Raven Press.

KIRINO, T. (1982). Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain Res.*, **239**, 57–69.

KUCHARCZYK, J., MINTOROVITCH, J., MOSELEY, M.E., ASGARI, H.S., SEVICK, R.J., DERUGIN, N. & NORMAN, D. (1991). Ischemic brain damage: reduction by sodium-calcium channel modulator RS-87476. *Radiology*, **179**, 221–227.

MAGNONI, M.S., GOVONI, S., BATTAINI, F. & TRABUCCHI, M. (1988). L-type calcium channels are modified in rat hippocampus by short-term experimental ischaemia. *J. Cereb. Blood Flow Metab.*, **8**, 96–99.

MCBEAN, D.E., WINTERS, V., WILSON, A.D., OSWALD, C.B. & ARMSTRONG, J.M. (1995a). Neuroprotective efficacy of the ion modulator lifarizine (RS-87476) in a rodent model of global cerebral ischaemia. *Br. J. Pharmacol.*, **14**, 331P.

MCBEAN, D.E., WINTERS, V., WILSON, A.D., OSWALD, C.B. & ARMSTRONG, J.M. (1995b). The ion channel modulator lifarizine (RS-87476) reduces the size of the infarct produced in the rat by the rose Bengal photochemical focal model of cerebral ischaemia. *Br. J. Pharmacol.*, **14**, 332P.

MINAMISAWA, H., MELLERGARD, P., SMITH, M.L., BENGTSSEN, F., THEANDER, S., BORIS-MOLLER, F. & SIESJO, B.K. (1990). Preservation of brain temperature during ischemia in rats. *Stroke*, **21**, 758–764.

PATMORE, L., DUNCAN, G.P. & SPEDDING, M. (1989). Interaction of palmitoyl carnitine with calcium antagonists in myocytes. *Br. J. Pharmacol.*, **97**, 443–450.

PULSINELLI, W.A. & BRIERLEY, J.B. (1979). A new model of bilateral hemispheric ischemia in the unanesthetized rat. *Stroke*, **10**, 267–272.

PULSINELLI, W.A., BRIERLEY, J.B. & PLUM, F. (1982). Temporal profile of neuronal damage in a model of transient forebrain ischemia. *Ann. Neurol.*, **11**, 491–495.

RATAUD, J., DEBARNOT, F., MARY, V., PRATT, J. & STUTZMANN, J.-M. (1994). Comparative study of voltage-sensitive sodium channel blockers in focal ischaemia and electric convulsions in rodents. *Neurosci. Lett.*, **172**, 19–23.

SANGUINETTI, M.C., KRAFTE, D.S. & KASS, R.S. (1986). Bay K8644: voltage-dependent modulation of Ca channel current in heart cells. *J. Gen. Physiol.*, **88**, 369–392.

SIESJO, B.K. & WIELOCH, T. (1985). Cerebral metabolism in ischaemia: neurochemical basis for therapy. *Br. J. Anaesth.*, **57**, 47–62.

SMITH, M.-L., AUER, R.N. & SIESJO, B.K. (1984). The density and distribution of ischemic brain injury in the rat following 2–10 min of forebrain ischemia. *Acta Neuropathol., (Berl)*, **64**, 319–332.

SNEDECOR, G.W. & COCHRAN, W.G. (1976). One way classification analysis of variance. In *Statistical Methods*, 6th ed. pp. 258–298. Ames, Iowa, U.S.A.: Iowa State Univ. Press.

SPEDDING, M. (1985). Activators and inactivators of Ca^{++} channels: new perspectives. *J. Pharmacol., (Paris)*, **16**, 319–343.

SPEDDING, M., KILPATRICK, A.T. & ALPS, B.J. (1989). Activators and inactivators of calcium channels: effects in the central nervous system. *Fundam. Clin. Pharmacol.*, **3**, 3s–29s.

SQUIRE, I.B., LEES, K.R., PRYSE-PHILLIPS, W., KERTESZ, A. & BAMFORD, J. (1994). Efficacy and tolerability of lifarizine in acute ischaemic stroke. Proc. *Second International Conference on Neuroprotective Agents: Clinical and Experimental Aspects*, Bolton Landing, Lake George, NY, U.S.A., 31st July–3rd August.

SUZUKI, R., YAMAGUCHI, T., CHOH-LUH, L. & KLATZO, I. (1983b). The effects of 5-minute ischemia in Mongolian gerbils: II. Changes of spontaneous neuronal activity in cerebral cortex and CA_1 sector of hippocampus. *Acta. Neuropathol., (Berl)*, **60**, 217–222.

SUZUKI, R., YAMAGUCHI, T., KIRINO, T., ORZI, F. & KLATZO, I. (1983a). The effects of 5-minute ischemia in Mongolian gerbils: 1. Blood-brain barrier, cerebral blood flow, and local cerebral glucose utilization changes. *Acta. Neuropathol., (Berl)*, **60**, 207–216.

WELSH, F.A. & HARRIS, V.A. (1991). Postischemic hypothermia fails to reduce ischemic injury in gerbil hippocampus. *J. Cereb. Blood Flow Metab.*, **10**, 617–620.

WELSH, F.A., SIMS, R.E. & HARRIS, V.A. (1990). Mild hypothermia prevents ischemic injury in gerbil hippocampus. *J. Cereb. Blood Flow Metab.*, **10**, 557–563.

ZOLA-MORGAN, S., SQUIRE, L.R. & AMARAL, D.G. (1986). Human amnesia and the medial temporal region: enduring memory impairment following a bilateral lesion limited to field CA 1 of the hippocampus. *Neurosci.*, **6**, 295–296.

(Received January 10, 1995
Revised May 9, 1995
Accepted May 10, 1995)