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Effects of Ca^{2+} and Na^{+} channel inhibitors in vitro and in global cerebral ischaemia in vivo

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Abstract

In the present study we have examined the effects of the small organic molecules: NNC 09-0026 ((−)-trans-1-butyl-4-(4-dimethylaminophenyl)-3-[(4-trifluoromethyl-phenoxy)methyl] piperidine dihydrochloride); SB 201823-A (4-[2-(3,4-dichlorophenoxy)ethyl]-1-pentyl piperidine hydrochloride); NS 649 (2-amino-1-(2,5-dimethoxyphenyl)-5-trifluoromethyl benzimidazole); CNS 1237 (*N*-acenaphthyl-*N′*-4-methoxynaphth-1-yl guanidine) and riluzole on human ω-conotoxin sensitive N-type voltage-dependent Ca^{2+} channel currents (I_{Ca}) expressed in HEK293 cells, on Na^{+} channel currents (I_{Na}) in acutely isolated cerebellar Purkinje neurones in vitro and in the gerbil model of global cerebral ischaemia in vivo. Estimated IC_{50} values for steady-state inhibition of I_{Ca} were as follows; NNC 09-0026, 1.1 μM; CNS 1237, 4.2 μM; SB 201823-A, 11.2 μM; NS 649, 45.7 μM and riluzole, 233 μM. Estimated IC_{50} values for steady-state inhibition of Na^{+} channel currents were as follows: NNC 09-0026, 9.8 μM; CNS 1237, 2.5 μM; SB 201823-A, 4.6 μM; NS 649, 36.7 μM and riluzole, 9.4 μM. In the gerbil model of global cerebral ischaemia the number of viable cells (mean ± S.E.M.) per 1 mm of the CA1 was 215 ± 7 (sham operated), 10 ± 2 (ischaemic control), 44 ± 15 (NNC 09-0026 30 mg/kg i.p.), 49 ± 19 (CNS 1237 30 mg/kg i.p.), 11 ± 2 (SB 201823-A 10 mg/kg i.p.), 17 ± 4 (NS 649 50 mg/kg i.p.) and 48 ± 18 (riluzole 10 mg/kg i.p.). Thus NNC 09-0026, CNS 1237 and riluzole provided significant neuroprotection when administered prior to occlusion while SB 201823-A and NS 649 failed to protect. These results indicate that the Ca^{2+} channel antagonists studied not only inhibited human N-type voltage-dependent Ca^{2+} channels but were also effective blockers of rat Na^{+} channels. Both NNC 09-0026 and CNS 1237 showed good activity at both Ca^{2+} and Na^{+} channels and this may contribute to the observed neuroprotection.

Keywords: HEK293 cell; Ca^{2+} channel; Na^{+} channel; NNC 09-0026; CNS 1237; SB 201823-A; NS 649; Riluzole; Cerebral ischaemia

1. Introduction

Although cerebral ischaemia is of major clinical importance in stroke and cerebrovascular disorders, the exact mechanism of ischaemia-induced neuronal cell death remains to be elucidated. It is thought that during ischaemia, the lack of energy to the brain may depolarize neurones and result in large increases in neurotransmitters such as glutamate, aspartate, dopamine and serotonin (Globus et al., 1988; Siesjö, 1992a,b). Glutamate, through an action on *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, allows Ca^{2+} to enter the cell (Watkins and Olverman, 1987; Choi, 1992; McCulloch, 1992). Glutamate can also act on metabotropic receptors leading to the production of diacylglycerol and inositol tris-phosphate, which upon activation of enzymes leads to the release of Ca^{2+} from intracellular stores (Watkins and Olverman, 1987; Choi, 1992). Ca^{2+} also enters neurones through voltage-dependent Ca^{2+} channels which open in response to cellular depolarization (Siesjö, 1992a,b). The net result of these various mechanisms by which Ca^{2+} concentrations are elevated is a Ca^{2+} 'overload' which leads to activation of proteases, nucleases, phospholipases, nitric oxide synthase and other degradative enzymes that lead to free radical production and cell death (Siesjö, 1992a,b).

Several studies have indicated that many compounds acting at excitatory amino acid receptors have beneficial effects against ischaemic insults (Gill et al., 1987; Grotta et al., 1990; Sheardown et al., 1990; Bullock et al., 1994).

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For example, NMDA receptor antagonists are neuroprotective in animal models of global and focal cerebral ischaemia (Gill et al., 1987; Boast et al., 1988; Grotta et al., 1990; McCulloch, 1992; Park et al., 1992; O'Neill et al., 1996) and recent studies have focused on the neuroprotective actions of AMPA receptor antagonists in animal models of cerebral ischaemia (Sheardown et al., 1990; Judge et al., 1991; Le Peillet et al., 1992; Sheardown et al., 1993a; Bullock et al., 1994; Gill, 1994). However, several early studies reported neuroprotection with compounds such as (S)-emopamil, which belong to the phenylalkylamine class of Ca^{2+} channel inhibitors, in animal models of cerebral ischaemia (Nakayama et al., 1988; Lin et al., 1990; Morikawa et al., 1991). In addition, other L-type Ca^{2+} antagonists such as nimodipine and nicardipine have been shown to display neuroprotective effects in some (Alps, 1992; Rami and Krieglstein, 1994) but not all animal models of ischaemia (Gomi et al., 1995).

At least 5 subtypes of high threshold, L, N, P, Q and R, and one type of low threshold, the T-type, voltage-dependent Ca^{2+} channel have been described (Tsien et al., 1995). The availability of several synthetic conopeptides has provided an opportunity to evaluate the therapeutic potential of selective blockade of N-type Ca^{2+} channels in a variety of pathological conditions including cerebral ischaemia. A single bolus intravenous administration of ω-conotoxin MVIIA (SNX-111) provided protection even when administered 24 h after the ischaemic insult (Valentino et al., 1993). Similar effects were observed in two other studies (Smith and Siesjö, 1992; Zhao et al., 1994). SNX-111 has also been found to be highly effective in reducing the neocortical infarct volume in rat models of focal ischaemia, both when administered during the occlusion (Takizawa et al., 1995) and after the ischaemic episode (Buchan et al., 1994). Buchan et al. (1994) administered SNX-111 at 5 mg/kg i.v. either 6 or 24 h after 10 min of 4-vessel occlusion and found protection in both cases. The group also showed protection with the same dose in a rat model of focal ischemia. However in contrast, ω-conotoxin MVIIC (SNX-230) which also blocks the other types of Ca^{2+} channel (P and Q type) in addition to N-type channels did not provide neuroprotection in the rat model of global ischaemia (Valentino et al., 1993).

More recent studies have reported neuroprotective effects with smaller nonpeptide compounds that inhibit neuronal Ca^{2+} channels. It has been reported that NNC 09-0026 ((−)-trans-1-butyl-4-(4-dimethylaminophenyl)-3-[(4-trifluoromethyl-phenoxy)methyl] piperidine dihydrochloride) inhibits neuronal voltage-dependent Ca^{2+} channels without cardiovascular effects and provides neuroprotective effects in animal models of cerebral ischaemia (Sheardown et al., 1993b; Barone et al., 1994). SB 201823-A (4-[2-(3,4-dichlorophenoxy)ethyl]-1-pentyl piperidine hydrochloride) is another nonpeptide Ca^{2+} channel blocker that exhibits broad activity against neuronal Ca^{2+} channels and at 10 mg/kg i.p. protected against global ischaemia-induced brain damage in the gerbil when administered 30 min after occlusion (Benham et al., 1993). More recent studies have reported that SB 201823-A antagonises Ca^{2+} currents in rat central neurones and reduces the infarct volume in rat and mouse models of focal ischaemia (Barone et al., 1995) and that another neuronal Ca^{2+} antagonist, NS-649 (2-amino-1-(2,5-dimethoxyphenyl)-5-trifluoromethyl benzimidazole, has provided protective effects in the mouse model of focal cerebral ischaemia (Varming et al., 1996). Several investigators have also examined the role played by Na^{+} channel blockers in ischaemic brain injury (Malgouris et al., 1989; Meldrum et al., 1992; Taylor and Meldrum, 1995). Riluzole (2-amino-6-trifluoromethoxybenzothiazole) was protective when administered at either 4 or 8 mg/kg i.p. 0.5, 4.5, 24 and 28 h after 6 min of occlusion in the gerbil (Pratt et al., 1992).

Due to the apparent specific effects of SNX-111 (ω-conotoxin MVIIA) on rat and human N-type voltage-dependent Ca^{2+} channels and the neuroprotective effects of this agent in vivo, we have examined the Ca^{2+} channel antagonist activity of NNC 09-0026, CNS 1237, NS 649, SB 201823-A and riluzole on human N-type Ca^{2+} channels expressed in HEK293 cells. In addition, we have performed a parallel study to examine the effects of these compounds on TTX-sensitive Na^{+} channels in acutely isolated cerebellar Purkinje neurons. Finally for comparison we also evaluated the neuroprotective properties of these compounds in the gerbil model of global cerebral ischaemia and compared their activity to the neuroprotectants (3S, 4aR, 6R, 8aR)-6-[2-(1(2)H-tetrazole-5-yl)]decahydroisoquinoline-3-carboxylic acid (LY293558, an AMPA receptor antagonist) and (+)-5-methyl-10, 11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801, an NMDA receptor antagonist).

2. Materials and Methods

2.1. Electrophysiology

2.1.1. Cell culture

HEK293 cells were maintained in DMEM (Gibco #320-1965AJ) defined, supplemented with bovine calf serum (5.5%) (Hyclone #A-2151-L), 100 U/ml penicillin G and 100 μg/ml streptomycin (Gibco #15140-015). HEK293 cells which had been stably transfected with $\alpha_{1B-1}\alpha_{2b}\beta_{1c}$ subunits cells (G1A1 cells) were transfected with pCMVα2bδ (10 μg), PSV2Hyg (1 μg) and pUC19 (9 μg) (C2D7 cells). (Williams et al., 1992; Sutton et al., 1996) A colony selected on the basis of high expression α2bδ using mRNA expression analysis and [^{125}I]ω-CgTx GVIA binding were maintained in plastic Petri dishes in a Dulbecco's modified Eagle's medium (Gibco) containing 5% defined, supplemented with bovine serum (Hyclone) plus penicillin G (100 U/ml), streptomycin sulphate (100

μg/ml), geneticin (500 μg/ml) and hygromycin B. One day prior to recording, cells were dissociated from the monolayer either mechanically or in Ca^{2+} free media. They were then centrifuged and replated onto poly-L-lysine coated glass coverslips (10 μg/ml) in the original media. Cells were used the following day.

2.1.2. Purkinje cell isolation

Purkinje cells were isolated according to a modification of the method of Mintz et al. (1992). Briefly, the cerebella were isolated from rats aged 6–11 days. Cerebellum vermi were dissected and cut into 0.5–1 mm^3 pieces and incubated at 37°C for 6 min in a solution consisting of 1 mg/ml protease XXIII (Sigma) in 82 mM Na_2SO_4, 30 mM K_2SO_4, 5 mM $MgCl_2$, 2 mM HEPES and 10 mM glucose (pH 7.4). These were then centrifuged and transferred to a solution containing 1 mg/ml BSA (Sigma), 1 mg/ml trypsin inhibitor (Sigma), 82 mM Na_2SO_4, 30 mM K_2SO_4, 5 mM $MgCl_2$, 10 mM Hepes and 10 mM glucose (pH 7.4). Cells were then dissociated by tritiation and plated onto poly-L-lysine coated glass coverslips (50 μg/ml). Purkinje neurons were identified morphologically by their large cell bodies (15–25 μm).

2.1.3. Whole-cell patch clamp

The tight seal whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) was used to record transmembrane Ca^{2+} currents (I_{Ca}) or Na^+ currents (I_{Na}). Cells were mounted in a perfusion chamber and thoroughly rinsed with buffer. The majority of HEK293 cells examined were less than 20 μm in diameter and spherical in appearance one day after replating. Currents were recorded using a List EPC-7 or Axopatch 1D amplifier, were filtered by an 8-pole low-pass Bessel filter and stored on computer. Linear leak corrections were performed using a P/N protocol. Series resistance compensation between 40 and 80% was applied. All experiments were performed at room temperature.

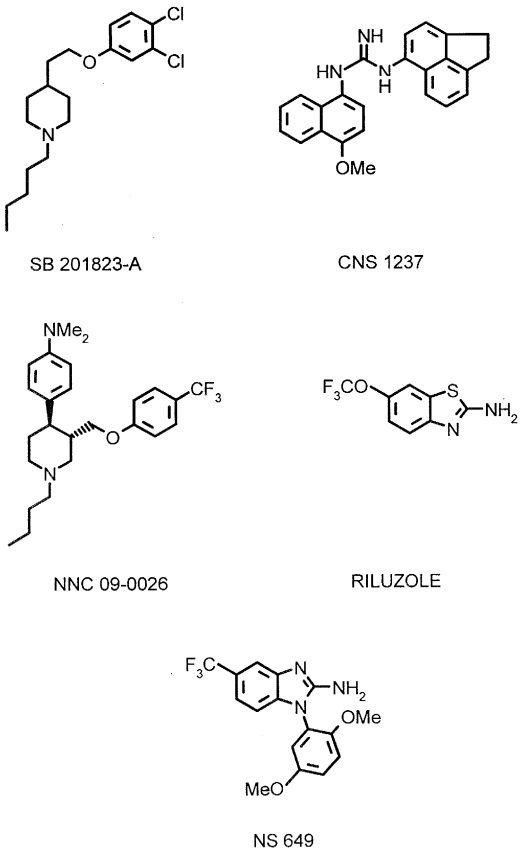

Fig. 1. Structures of NNC 09-0026, SB 201823-A, NS 649, CNS 1237 and riluzole.

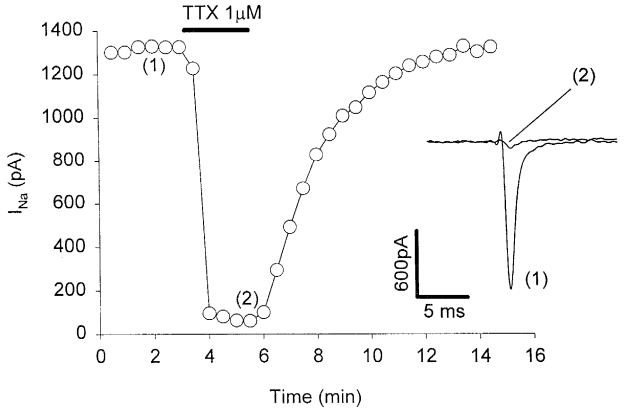

Fig. 2. Representative example of the time-dependent inhibition of acutely isolated Purkinje cell Na^+ currents by TTX (1 μM). Currents were evoked from a holding potential (V_h) of −70 mV to a test potential (V_t) of 0 mV (duration 50 ms) every 30 s. The inset shows individual current records for the time points indicated: (1) Control and (2) during application of TTX.

2.1.4. Extracellular solutions

Control buffer solutions were composed of (in mM): NaCl, 138; $CaCl_2$, 5; $MgCl_2$, 1; KCl, 5; HEPES, 10; glucose, 10; adjusted to pH 7.4 with NaOH. Voltage-clamp experiments were performed with cells perfused with solutions containing (in mM): tetraethylammonium chloride (TEACl), 143; $CaCl_2$, 5; $MgCl_2$, 1; HEPES, 10; glucose, 10; pH adjusted to 7.4 with tetraethylammonium hydroxide., when isolating for VDCC. Cerebellar Purkinje I_{Na} currents were measured in solutions containing (in mM): NaCl, 70; TEACl, 70; $CaCl_2$, 5; $MgCl_2$, 1; KCl, 2.5; HEPES, 10; glucose, 10; adjusted to pH to 7.4 with NaOH and in the presence of 100 μM $CdCl_2$.

2.1.5. Pipette solutions

Solutions for voltage-clamp experiments contained (in mM): CsCl, 135; $MgCl_2$, 1; HEPES, 10; di-Tris phosphocreatinine, 14; MgATP, 3.6; 50 U/ml creatinine phosphokinase; BAPTA, 15, adjusted to pH 7.1 with CsOH.

2.1.6. Analysis

Curve fitting to data points was performed using the following equation; $y = 100(D^n/D^n + K_d{}^n)$ where $y =$ the % inhibition, $D =$ antagonist concentration and n is the slope.

2.1.7. Materials

All reagents were of the highest commercial grade. ω-CgTx-GVIA was from Peninsular Laboratories. NNC 09-0026 ((−)-trans-1-butyl-4-(4-dimethylaminophenyl)-3-[(4-trifluoromethylphenoxy)methyl] piperidine dihydrochloride), SB 201823-A (4-[2-(3,4-dichlorophenoxy)ethyl]-1-pentyl piperidine hydrochloride), NS 649 (2-amino-1-(2,5-dimethoxyphenyl)-5-trifluoromethyl benzimidazole) and CNS 1237 (*N*-acenaphthyl-N′-4-methoxynaphth-1-yl guanidine) were synthesized at Lilly Research Centre. Compounds were made up as stock solutions in DMSO to give final concentrations in solutions which limited the DMSO concentration to $\leq 0.2\%$.

2.2. Ischaemia studies

2.2.1. Animals and surgery

Male Mongolian gerbils (Bantin and Kingman) at least 3 months old and weighing in excess of 60 g were used. The animals were maintained in standard lighting conditions and food and water were available ad libitum. The animals were anaesthetised with a 5% halothane/oxygen mixture and maintained using 2% halothane delivered with oxygen at 1 l/min via a face mask throughout the operation. Through a midline cervical incision, both common carotid arteries were exposed and freed from surrounding connective tissue. In animals to be rendered ischaemic both common carotid arteries were clamped for 5 min. At the end of the occlusion period 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. Throughout surgery body temperature was maintained at 37°C using a 'K-TEMP' temperature controller/heating pad (International Market Supply) and brain temperatures were maintained using a heating lamp. After surgery the animals were placed in a four compartmental thermacage (Beta Medical and Scientific) which maintained the environmental temperature at 28°C and rectal temperatures were measured for a 6 h period after occlusion.

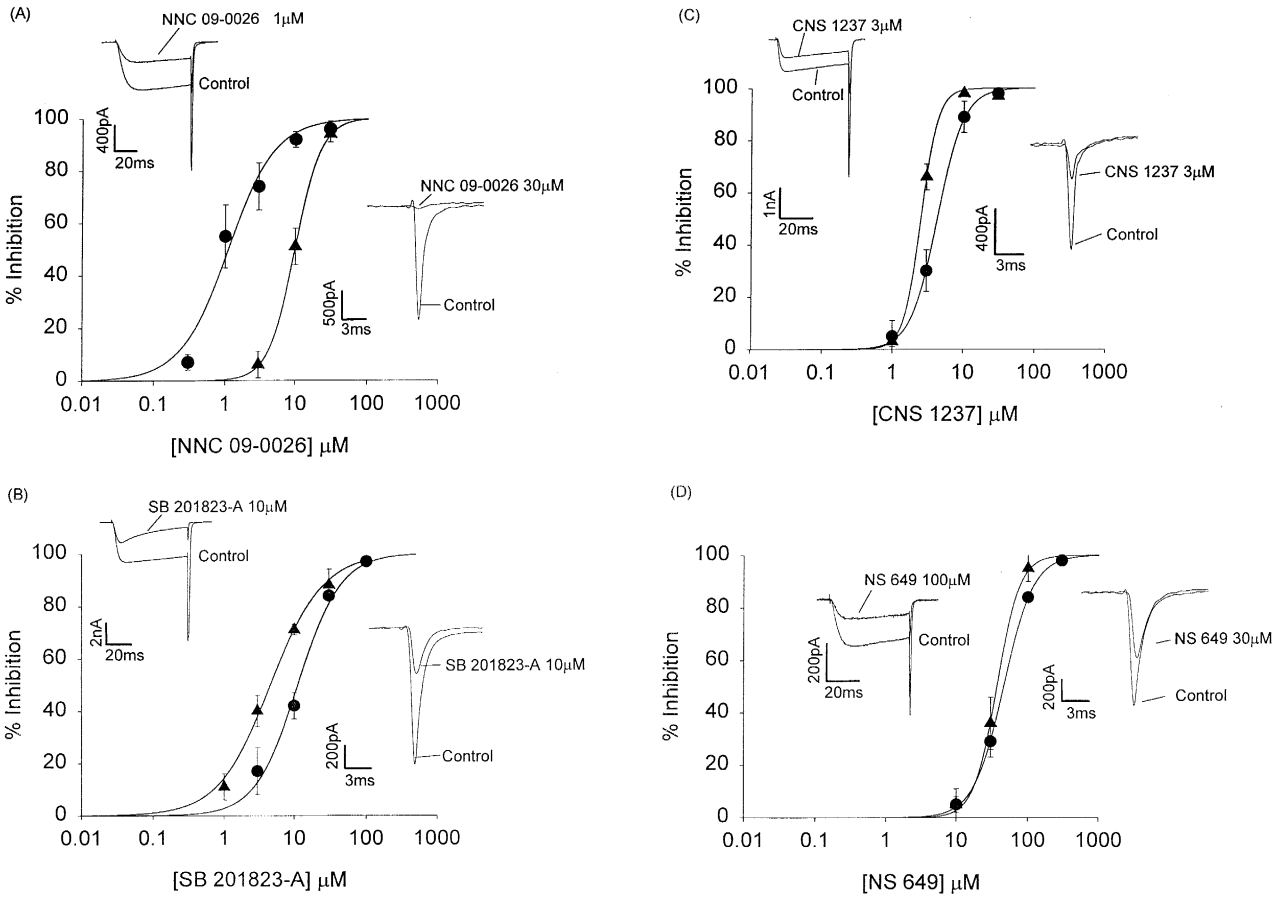

Fig. 3. Effects of compounds on Ca^{2+} and Na^+ channels determined using whole-cell voltage clamp electrophysiology. Concentration–response curves showing block at the human N-type VDCC and block of Na^+ channel currents recorded from acutely isolated Purkinje cells. Data points represent mean values ± s.e. mean for between 3 and 6 observations at each concentration. (A) NNC09-0026 at the N-type VDCC (●) and Purkinje cell Na^+ channel currents (▲). (B) SB201823-A at the N-type VDCC (●) and Purkinje cell Na^+ channel currents (▲). (C) CNS1237 at the N-type VDCC (●) and Purkinje cell Na^+ channel currents (▲). (D) NS 649 at the N-type VDCC (●) and Purkinje cell Na^+ channel currents (▲). The insets for each figure show example traces of currents inhibited by the compounds.

2.2.2. Treatment groups

In the present studies all the doses selected and the frequency and timing of the doses were based on previous work in the literature and also the tolerability on the compounds in animals. NNC 09-0026 was administered at (i) 30 mg/kg i.p. at −30 min, +24 h and +48 h post-occlusion or (ii) 30 mg/kg i.p. at 0, +24 h and +48 h post-occlusion. CNS 1237 (30 mg/kg i.p.), SB 201823-A (10 mg/kg i.p.) and NS 649 (50 mg/kg i.p.) were administered at 30 min before and 2 h 30 min after occlusion. Riluzole (6 or 10 mg/kg i.p.) was administered 30 min before, 2 h 30 min, 24 and 48 h after occlusion. LY293558 was administered at 20 mg/kg i.p. immediately after occlusion followed by two further doses of 10 mg/kg i.p. at 3 and 6 h post occlusion and MK-801 was administered at 2 mg/kg i.p. immediately post-occlusion.

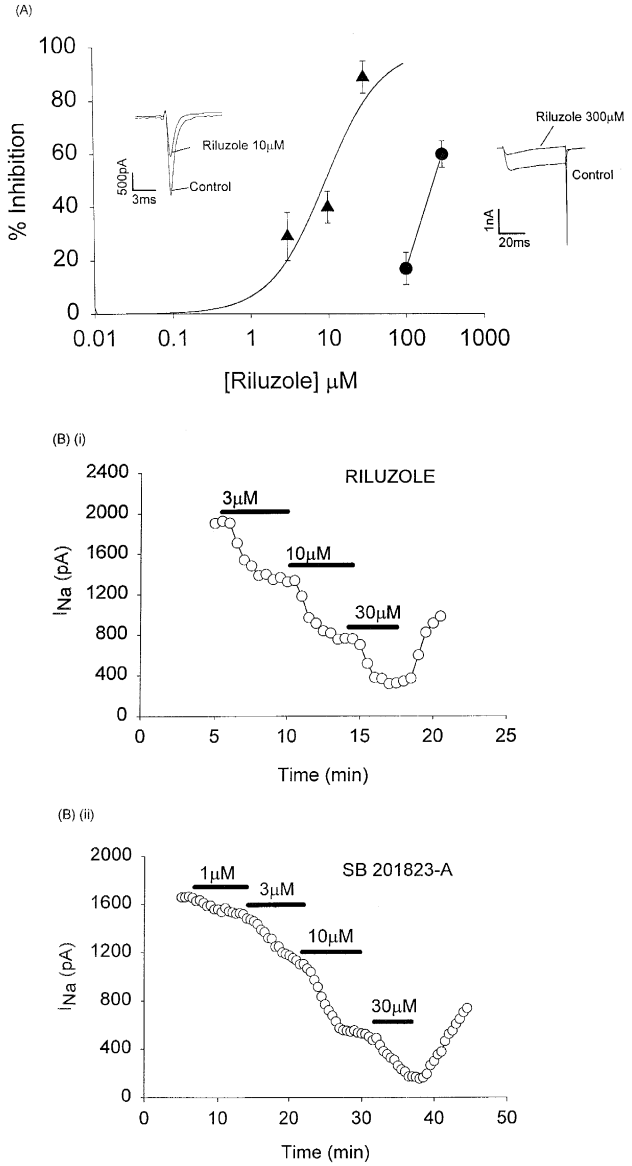

Fig. 4. (A) Concentration–response curves showing block by riluzole of the human N-type VDCC (●) and block of Na^+ channel currents (▲) recorded from acutely isolated Purkinje cells. Data points represent mean values ± s.e. mean for between 3 and 6 observations at each concentration. (B) Representative time-course of block of the Na^+ channel currents recorded from acutely isolated Purkinje cells by riluzole (i) and SB 201823-A (ii).

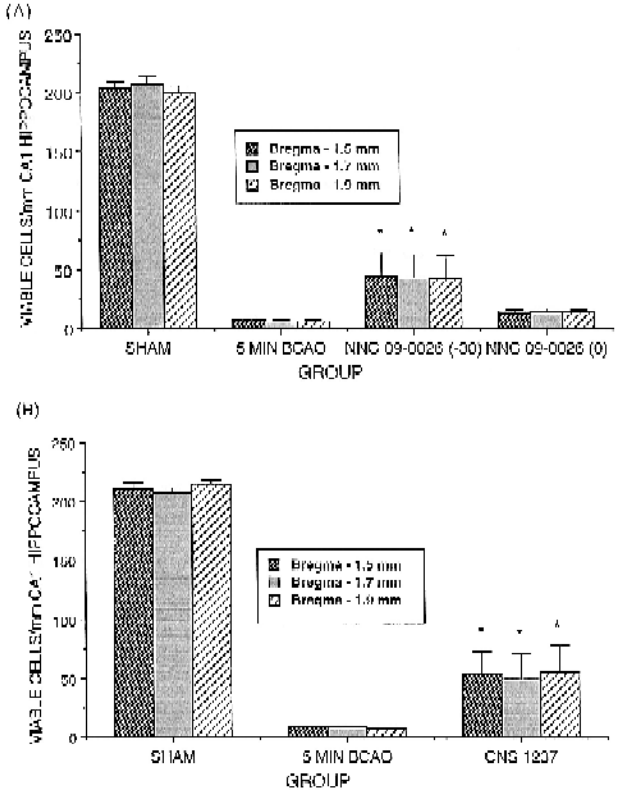

Fig. 5. The effects of NNC 09-0026 (A) and CNS 1237 (B) on the neuronal density in the CA1 region of the hippocampus 5 days after 5 min bilateral carotid artery occlusion. NNC 09-0026 was administered at 30 mg/kg either 30 min before or immediately after occlusion followed by two further doses at 24 and 48 h post-occlusion. CNS 1237 was administered at 30 mg/kg either 30 min before and 2 h 30 min after occlusion. Results are expressed as mean ± S.E.M. viable cells/mm CA1 hippocampus ($n = 8$ animals per group). 5 min of occlusion caused a severe loss in neuronal cells in the CA1 region ($P < 0.001$). NNC 09-0026 and CNS 1237 provided significant neuroprotection ($P < 0.05$) against the ischaemia-induced cell death when administration was initiated 30 min prior to occlusion. NNC 09-0026 failed to provide any neuroprotection when administration was delayed until immediately after occlusion. Student's *t*-test.

2.2.3. 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 1.5–1.9 mm caudal to the bregma in the anterior hippocampus using a sledge microtome (Leitz 1400). 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.

2.2.4. Statistics

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

3. Results

The structures of NNC 09-0026, CNS 1237, NS 649, SB 201823-A and riluzole are shown in Fig. 1.

3.1. Electrophysiology

Voltage-dependent Ca^{2+} channel currents were recorded in HEK293 cells transfected with the human N-type Ca^{2+} channel (C2D7 cells) (Williams et al., 1992, Bleakman et al., 1995; Sutton et al., 1997). We have previously demonstrated that the human N-type Ca^{2+} channel is inhibited by ω-conotoxin GVIA, MVIIA and MVIIC (Grantham et al., 1994; Bleakman et al., 1995; Bath et al., 1996). Voltage-dependent Na^+ channels were recorded in acutely isolated rat cerebellar Purkinje neurons. Purkinje cell soma recordings from organotypic cerebellar cultures have previously shown properties of type II Na^+ channels (Gahwiler and Llano, 1989). In the present study application via bath perfusion of 1 μM TTX resulted in reversible 98 ± 3% ($n = 3$) inhibition of the Na^+ current (Fig. 2).

The inhibitory effects of NNC 09-0026, CNS 1237, NS 649, SB 201823-A and riluzole were examined on Ca^{2+} currents in C2D7 cells. Currents were evoked by depolarising from a holding potential of −90 mV to a test potential of 10 mV for 50 ms every 30 s. Application of compounds to cells via bath perfusion resulted in concentration-dependent inhibition of the voltage dependent Ca^{2+} currents (VDCC) (Fig. 3A–D). Steady state inhibition was measured and data pooled for cells in which the compounds had been examined. Approximate IC_{50} values for these compounds were estimated from the graphs as: NNC 09-0026, 1.1 μM; CNS 1237, 4.2 μM; SB 201823-A, 11.2 μM and NS 649, 45.7 μM. Reversible block of the Ca^{2+} channel currents was observed in all cases although the extent of reversibility varied between compounds.

The effects of the Ca^{2+} channel antagonists were also examined on Na^+ channel currents in Purkinje cells. Currents were evoked by depolarisation from a holding potential of −70 mV to a test potential of 0 mV for 50 ms every 30 s. Compounds were applied to the cells via bath perfusion and the steady state inhibition of currents measured. As with the Ca^{2+} currents NNC 09-0026, CNS 1237, SB 201823-A and NS 649 inhibited Na^+ channel currents to varying degrees in a concentration-dependent manner (Fig. 3A–D). Estimated IC_{50} values from the concentration–response curves were: NNC 09-0026, 9.8 μM; CNS 1237, 2.5 μM; SB 201823-A, 4.6 μM; NS 649, 36.7 μM.

As all of the Ca^{2+} channel antagonists tested also showed Na^+ channel activity we assessed the activity of riluzole which has been reported to have inhibitory effects at Na^+ channels (Tsai et al., 1987; Benoit and Escande, 1991). Riluzole was found to have only weak inhibitory effects at the human N-type Ca^{2+} channel with an inhibition of 17 ± 6% ($n = 3$ cells) at 100 μM and 60 ± 5% ($n = 6$ cells) at 300 μM (Fig. 4A). Riluzole inhibited Na^+ channels in cerebellar Purkinje cells in a concentration-dependent manner with an estimated IC_{50} value of 11.4 μM (Fig. 4A). Representative current inhibition for SB 201823-A and riluzole are shown in (Fig. 4B).

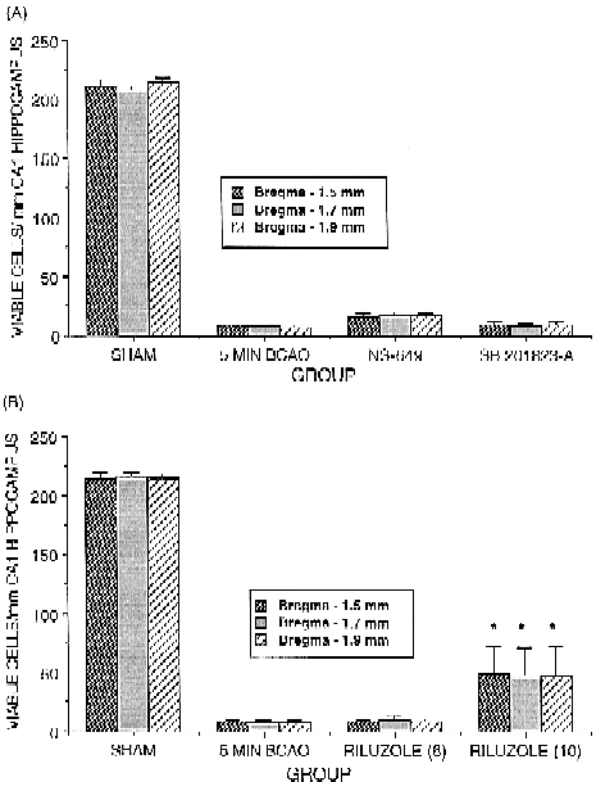

Fig. 6. The effects of NS-649 and SB 201823-A (A) and riluzole (B) on the neuronal density in the CA1 region of the hippocampus 5 days after 5 min bilateral carotid artery occlusion. NS-649 was administered at 50 mg/kg either 30 min before and 2 h 30 min after occlusion and SB 201823-A was administered at 10 mg/kg either 30 min before and 2 h 30 min after occlusion. Riluzole was administered at either 6 or 10 mg/kg 30 min before occlusion followed by three further doses at 2 h 30 min, 24 and 48 h after occlusion. Results are expressed as mean ± S.E.M. viable cells/mm CA1 hippocampus ($n = 8$ animals per group). 5 min of occlusion caused a severe loss in neuronal cells in the CA1 region ($P < 0.001$). Both NS-649 and SB 201823-A failed to provide any protection against the ischaemia-induced cell death. The 6 mg/kg dose of riluzole failed to protect, but the 10 mg/kg dose provided significant neuroprotection ($P < 0.05$) against the ischaemia-induced cell death. Student's *t*-test.

3.2. Ischaemia results

5 μm sections taken 1.5, 1.7 and 1.9 mm caudal to the bregma in the anterior hippocampus were examined under a microscope with grid lines. The pyramidal cell density was counted at three different stereotaxic levels in the CA1 region of the hippocampus and the results expressed as mean ± S.E.M. neuronal density per 1 mm CA1. % neuroprotection was calculated using the number of viable cells/mm CA1 hippocampus in drug treated, 5 min occluded and sham operated controls at 1.7 mm caudal to the bregma. The results indicated that there was severe loss of neurones in the CA1 region of the hippocampus of 5 min occluded animals. The neuronal death involved nearly all the pyramidal neurones and this neurodegeneration was not evident in any other forebrain region.

NNC 09-0026 protected (22%) against this ischaemia-induced cell death in the CA1 region when administered at 30 mg/kg i.p. 30 min before followed by two further doses at 24 and 48 h post-occlusion (Fig. 5A). The compound failed to protect when administration was delayed until immediately after occlusion. CNS 1237 provided similar (21%) protection when administered at 30 mg/kg i.p. 30 min before and 2 h 30 min post-occlusion (Fig. 5B). In contrast, NS 649 (50 mg/kg i.p.) and SB 201823-A (10 mg/kg i.p.) failed to provide any neuroprotection when administered 30 min before and 2 h 30 min after occlusion (Fig. 6A).

Riluzole provided no neuroprotection when administered at 6 mg/kg i.p. 30 min before and 2 h 30 min, 24 and 48 h after occlusion. However, when administered at 10 mg/kg i.p. using the same protocol riluzole provided significant (18%) protection (Fig. 6B). For comparison we also examined the effects of an NMDA antagonist (MK-801) and an AMPA antagonist (LY293558) in the model. MK-801 provided significant (24%) protection when dosed at 2 mg/kg i.p. immediately post-occlusion (Fig. 7). The AMPA antagonist LY293558 provided good (30%) neuroprotection when dosed at 20 mg/kg immediately post-occlusion, followed by two further doses of 10 mg/kg i.p. at 3 and 6 h post-occlusion (Fig. 7). For comparison purposes Table 1 summarizes the in vitro and in vivo data.

Table 1
Comparison of inhibitory actions of NNC 09-0026, SB 201823-A, NS 649, CNS 1237 and riluzole on human ω-conotoxin sensitive N-type voltage-dependent calcium channel currents (I_{Ca}) expressed in HEK293 cells, on sodium channel currents (I_{Na}) in acutely isolated cerebellar Purkinje neurones in vitro and in the gerbil model of global cerebral ischemia in vivo

Compound	I_{Ca} IC_{50} (μM)	I_{Na} IC_{50} (μM)	% Neuroprotection	Dose (mg/kg)
CNS1237	4.2	2.5	21	30
Riluzole	> 200	11.4	18	10
NCC 09-0026	1.1	9.8	22	30
NS 649	45.6	36.7	0	50
SB 201823-A	11.2	4.6	0	10

% neuroprotection was calculated using the number of viable cells/mm CA1 hippocampus from the drug treated animals, 5 min occluded and sham operated controls at 1.7 mm caudal to bregma. All compounds were administered via the i.p. route.

4. Discussion

4.1. Ca^{2+} channel and Na^{+} channel activities

In the present studies we have performed direct comparisons of the effects of several compounds reported as Ca^{2+}

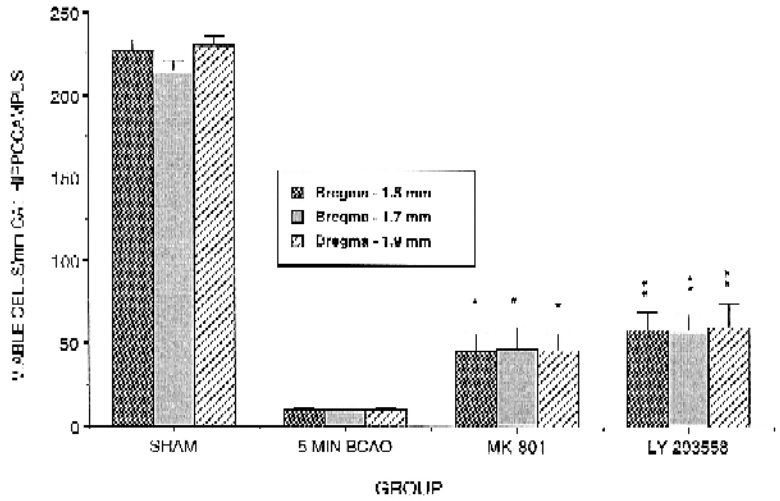

Fig. 7. The effects of MK-801 and LY293558 on the neuronal density in the CA1 region of the hippocampus 5 days after 5 min bilateral carotid artery occlusion. MK-801 was administered at 2 mg/kg immediately after occlusion and LY293558 was administered at 20 mg/kg immediately after occlusion followed by two further doses 10 mg/kg at 3 and 6 h after occlusion. Results are expressed as mean ± S.E.M. viable cells/mm CA1 hippocampus ($n = 8$ animals per group). 5 min of occlusion caused a severe loss in neuronal cells in the CA1 region ($P < 0.001$). MK-801 ($P < 0.05$) and LY 293558 ($P < 0.01$) provided good neuroprotection against the ischaemia-induced cell death when administered post-occlusion. Student's *t*-test.

channel antagonists on human N-type Ca^{2+} channels expressed in HEK293 cells and on TTX-sensitive Na^{+} channels in cerebellar Purkinje neurons. NNC 09-0026 has been reported to inhibit potassium-stimulated Ca^{2+} uptake into rat synaptosomes with an approximate IC_{50} value of 13 μM and inhibit dorsal root Ca^{2+} currents by 43% at a concentration of 10 μM (Barone et al., 1994). Benham et al. (1993) have reported that SB 201823-A blocked Ca^{2+} currents in dorsal root ganglion cells and superior cervical ganglion neurons with an IC_{50} value of approximately 5 μM. Barone et al. (1995) found that SB 201823-A was able to block Ca^{2+} channel currents in cultured hippocampal and cerebellar granule cell bodies with approximate IC_{50} values between 5 and 20 μM. NS-649 has previously been examined on embryonic chick dorsal root ganglia and an IC_{50} value of 32 μM was reported (Varming et al., 1996). CNS 1237 has been shown to block Ca^{2+} influx into rat synaptosomes with an approximate IC_{50} value of 2 μM and Na^{+} channel activity (type II expressed in CHO cells) with an approximate IC_{50} value of 1.3 μM (Reddy et al., 1995). The present results indicate that these compounds inhibit the human N-type Ca^{2+} channel with the following rank order of potency: NNC 09-0026 (1.1 μM) > CNS 1237 (4.2 μM) > SB 201823-A (11.2 μM) > NS 649 (45.6 μM). These results are in general agreement with previous literature reports showing that the above compounds block Ca^{2+} channels in a variety of cell types. However, many of the previous studies were carried out in preparations with mixed populations of Ca^{2+} channels and invariably under different recording conditions (stimulus paradigms and ionic conditions) which makes direct comparisons of relative activity problematic.

Previous studies have indicated that CNS 1237 may be a use-dependent blocker of Na^{+} and Ca^{2+} channels (Goldin et al., 1995) although in the present study we have not examined the use-dependent nature of block of any of the compounds tested. In addition, it has been reported that SB 201823-A has effects on Na^{+} channels in dorsal root ganglion neurons with a 47% inhibition at 20 μM. Our present findings suggest that SB 201823-A has a marked inhibitory effect at Na^{+} channels in rat cerebellar Purkinje neurons (IC_{50} value of 4.6 μM). We also observed that NNC 09-0026 and CNS 1237 also blocked Na^{+} channels, whereas NS 649 provided little block of Na^{+} channels in isolated Purkinje cells. Riluzole was the only compound that showed good selectivity for Na^{+} channels over Ca^{2+} channels with weak inhibitory effects on the N-type Ca^{2+} channel even at high concentrations (> 100 μM) and an IC_{50} of approximately 9.4 μM on Na^{+} channels.

4.2. Anti-ischaemic properties

In the present studies we evaluated several neuronal Ca^{2+} channel antagonists in the gerbil model of cerebral ischaemia. The doses selected were based on previously published work and tolerability in animals. NNC 09-0026 (30 mg/kg i.p.) provided 22% neuroprotection when administration was initiated 30 min before occlusion followed by two further doses at 24 and 48 h post-occlusion. Sheardown et al. (1993b) have reported that NNC 09-0026 (30 mg/kg i.p.) protected in the gerbil when administered 30 min, 24 and 48 h post-occlusion, but no neuroprotection when the compound (30 mg/kg i.p.) was administered once 30 min post-occlusion. In the present studies we found no neuroprotection with NNC 09-0026 when administered at 30 mg/kg i.p. immediately after occlusion followed by two further doses of 30 mg/kg at 24 and 48 h post-occlusion. NNC 09-0026 (30 mg/kg i.v.) administered slowly over a 1 h period beginning 30 min postischaemia has also been reported to reduce the infarct volume in a rat model of focal ischaemia (Barone et al., 1994).

CNS 1237 provided some neuroprotection (21%) against the ischaemia-induced cell death in the CA1 region of the hippocampus. Previous studies have shown that CNS 1237 is a use-dependent Na^{+} and Ca^{2+} channel blocker (Goldin et al., 1995). CNS 1237 produced a reduction in infarct volume when dosed as a bolus of 3 mg/kg i.v. followed by an infusion of 0.75 mg/kg/h for 4 h (Goldin et al., 1995).

In the present studies SB 201823-A administered at 10 mg/kg 30 min before and 2 h 30 min after occlusion failed to provide any neuroprotection. However, previous studies have reported that SB 201823-A administered at 10 mg/kg i.p. at 30 min post-occlusion attenuated ischaemia-induced hyperactivity and protected the CA1 pyramidal cells in the hippocampus following 8 min of bilateral carotid artery occlusion in the gerbil (Benham et al., 1993). Other studies have indicated that SB 201823-A antagonises Ca^{2+} currents in central neurones and reduces the infarct volume in rat and mouse models of focal ischaemia (Barone et al., 1995). However, a recent study reported that MVIIA (300 nmol/l) protected, but SB 201823-A (3 μmol/l) failed to protect against hypoxia-induced neurodegeneration in organotypic hippocampal-slice cultures (Pringle et al., 1996). The authors also observed that higher doses of SB 201823-A were directly toxic.

NS 638 (2-amino-1-(4-chlorobenzyl)-5-trifluoromethyl benzimidazole) is a nonpeptide Ca^{2+} antagonist that has been reported to reduce the infarct volume by 48% in a mouse model of focal ischaemia (Moller et al., 1995). However NS 638 administered at 30 mg/kg i.p. 1, 4 and 24 h post-occlusion failed to protect against CA1 damage in the gerbil model of global ischaemia (Moller et al., 1995). NS 649 is another compound from this series that has also shown protective effects in the mouse model of focal ischaemia (Varming et al., 1996). In the present studies NS 649 at 50 mg/kg i.p. (dosed 30 min before and again at 2 h 30 min after occlusion) failed to provide any protection against the ischaemia-induced damage in the CA1 hippocampal region of the gerbil brain.

In the present studies 6 mg/kg of riluzole failed to

protect against the ischaemia-induced damage in the hippocampus. However, the 10 mg/kg dose provided some neuroprotection (18%). Previous studies have reported riluzole was protective when administered at either 4 or 8 mg/kg i.p. 0.5, 4.5, 24 and 28 h after 6 min of occlusion in the gerbil (Pratt et al., 1992). The authors suggested that the mechanism of protection may be by maintaining Na^+ channels in their inactivated state, which in turn leads to a decrease in the release of excitatory amino acids.

The NMDA receptor antagonist MK-801 and the AMPA receptor antagonist LY293558 provided good neuroprotection (24% and 30%, respectively) when administered post-occlusion. We have also found that if administration is initiated before occlusion MK-801 provided 25% and LY293558 provided 56% protection (unpublished results). Several studies have reported that MK-801 is neuroprotective in animal models of cerebral ischaemia (Gill et al., 1987, McCulloch, 1992). More recent studies have also reported that LY293558 is neuroprotective in a cat model of focal ischaemia (Bullock et al., 1994). From the present studies it is clear that MK-801 and LY293558 provide greater neuroprotection than the other compounds tested.

The present results indicate that the compounds tested were able to block human N-type Ca^{2+} channels to varying degrees, however none showed significant selectivity for Ca^{2+} channels over Na^+ channels. In particular, NNC 09-0026 and CNS 1237 exhibited good activity at both Ca^{2+} and Na^+ channels in vitro and also offered neuroprotection in the gerbil model of global ischaemia. This suggests that good activity at both Na^+ and Ca^{2+} channels may be responsible for the observed neuroprotection. Riluzole provided a similar degree of protection to NNC 09-0026 and CNS 1237 which may suggest that the ability of compounds to protect in this model may reside in activity at Na^+ channels. However, SB 201823-A had better Na^+ channel activity than riluzole under the present in vitro recording conditions and did not provide neuroprotection in our model. There may be several reasons for the lack of neuroprotection with SB 201823-A: (a) the compound may not cross the blood–brain barrier (or penetrate the brain poorly); (b) the half-life of the compound in the brain may be short (c) the compound may be unstable or metabolised quickly, etc. Clearly, pharmokinetic properties and the timing of dosing are important, for example NCC 09-0026 was protective when administered both before and after occlusion, but failed to show any neuroprotection when dosing was delayed until post-occlusion. Several of the compounds tested have high lipophilicity and although we have demonstrated that these compounds have Na^+ channel activity in addition to their Ca^{2+} channel activity we cannot rule out that some of the compounds also have activity at other receptors and this may contribute to the observed or lack of neuroprotection. The above point is best illustrated using riluzole as an example as it is possible that the neuroprotective effects of riluzole are not entirely due its Na^+ channel activity. A recent study has suggested that in addition to effects on Na^+ entry, riluzole also prevents NMDA receptor mediated responses via a pertussis toxin sensitive mechanism (Hubert et al., 1994). The mechanism(s) which are responsible for the observed neuroprotection by riluzole remains to be established. In relation to other compounds tested we have shown that MK-801 and LY293558 administered after occlusion provide greater neuroprotection than the other compounds tested. The emergence of potent and selective small organic Ca^{2+} channel antagonists without effects at Na^+ channels could assist in the clarification of which type of channel activity in a compound is important for neuroprotection.

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