ELSEVIER

European Journal of Pharmacology 411 (2001) 71–83

ejp

www.elsevier.nl/locate/ejphar

The effects of LY393613, nimodipine and verapamil, in focal cerebral ischaemia

Liesbeth Bogaert [a], Michael J. O'Neill [b], Jan Moonen [a], Sophie Sarre [a], Ilse Smolders [a], Guy Ebinger [c], Yvette Michotte [a,*]

[a] *Department of Pharmaceutical Chemistry and Drug Analysis, Pharmaceutical Institute, Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussels, Belgium*

[b] *Eli Lilly and Co. Ltd., Lilly Research Centre, Erl Wood Manor, Windlesham, Surrey GU20 6PH, UK*

[c] *Department of Neurology, University Hospital, Laarbeeklaan 101, 1090 Brussels, Belgium*

Received 29 June 2000; received in revised form 1 November 2000; accepted 7 November 2000

Abstract

This study evaluates the effects of *N*-{2-[bis (4-fluorophenyl)methoxy]ethyl}-1-butanamine hydrochloride (LY393613), a novel neuronal (N/P/Q-type) Ca^{2+} channel blocker, in ischaemia. For comparison, two commonly used L-type Ca^{2+} channel blockers; nimodipine and verapamil were also evaluated. Ischaemia was induced in freely moving rats by micro-injection of endothelin-1 near the middle cerebral artery. In vivo microdialysis, laser Doppler flowmetry and histology were used to monitor ischaemia. Administration of LY393613, before and after the insult, attenuated the ischaemia-induced glutamate release, but not the dopamine release. Both nimodipine and verapamil failed to affect transmitter releases significantly, when administered post-occlusion. None of the compounds tested, produced any significant change in striatal blood flow. Histology showed that ischaemic damage was significantly less in LY393613 pre-treated rats. In conclusion, LY393613, a neuronal N/P/Q-Ca^{2+} channel blocker, can attenuate ischaemic brain damage. The protective mechanism appears to be *mainly* the attenuation of the ischaemia-induced glutamate release, rather than its effect on cerebral hemodynamics.

Keywords: Ca^{2+} channel blocker; LY393613; Nimodipine; Verapamil; Ischaemia, focal; Neuroprotection

1. Introduction

Brain damage, secondary to ischaemia is thought to result from a cascade of interconnected pathological processes, including changes in cerebral blood flow as well as progressive alterations of ion homeostasis and cellular metabolism (Choi, 1992; Siesjö, 1992a,b). During ischaemia, the lack of energy to the brain may depolarize neurons, producing a large increase in neurotransmitters such as glutamate (Glu), aspartate, dopamine and serotonin (Globus et al., 1988; Siesjö, 1992a,b). These neurotransmitters can post-synaptically cause a Ca^{2+} influx into the cells, and also release Ca^{2+} from intracellular stores. The importance of this intracellular Ca^{2+} has been extensively studied, since a sustained elevated concentration of intracellular Ca^{2+} can activate degradative processes, and lead to cell necrosis and programmed cell death (Nitatori et al., 1995; Clapham, 1995; Nicotera et al., 1992). For example, the huge increase in intracellular Ca^{2+} in ischaemia causes the activation of proteases, nucleases, phospholipases, nitric oxide (NO) synthase and other degradative enzymes that lead to free radical production, mitochondrial degeneration and cell death (Siesjö, 1992a,b).

Consistent with the above mechanisms, several studies using cerebral ischaemia models, have reported neuroprotective actions of Ca^{2+} channel blockers (Alps, 1992) and Glu receptor antagonists (McCulloch, 1992). For example, L-type Ca^{2+} channel antagonists, such as (*S*)-emopamil,

* Corresponding author. Tel.: +32-2-477-4746; fax: +32-2-477-4113.
E-mail address: ymichot@minf.vub.ac.be (Y. Michotte).

0014-2999/01/$ - see front matter
PII: S0014-2999(00)00861-X

nimodipine and nicardipine have been shown to display neuroprotective effects in some (Alps, 1992; Lin et al., 1990; Morikawa et al., 1991; Rami and Krieglstein, 1994), but not all animal models of ischaemia (Gomi et al., 1995). However, many of these earlier molecules may have primarily an effect on the cerebral blood flow and blood pressure, and this may have been accountable for the observed neuroprotection.

Further studies demonstrated that the selective blockade of neuronal Ca^{2+} channels was neuroprotective. For example, the N-type blocker, ω-conotoxin MVIIA provided protection even when administered 24 h after global ischaemia (Valentino et al., 1993). Similar effects were observed in two other studies (Smith and Siesjö, 1992; Zhao et al., 1994). ω-Conotoxin MVIIA 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), as well as after the ischaemic episode (Buchan et al., 1994). However, these conotoxins being large peptides are not ideal as drug candidates. Therefore, small molecules that block neuronal Ca^{2+} channels would be better anti-ischaemic agents. *N*-{2-[bis (4-fluorophenyl)-methoxy]-ethyl}-1-butanamine hydrochloride, named LY393613, is a novel neuronal Ca^{2+} channel blocker, that inhibits human $\alpha 1A$, $\alpha 1B$ and $\alpha 1E$ Ca^{2+} channel subunits expressed in Human Embryonal Kidney (HEK) 293 cells, P-type Ca^{2+} channels in isolated cerebellar Purkinje cells and KCl-induced neurotransmitter release from synaptosomes (O'Neill et al., 1997). Hence, LY393613 can be classified as an N/P/Q-type Ca^{2+} channel blocker.

Although the combination of restoring blood flow and providing neuroprotection may be the most effective approach in acute ischaemic insults and considerable interest has been given to Ca^{2+} channel entry blockers in preventing and/or reversing pathological ischaemic damage, only few studies reported the effect of these drugs on hemodynamics and neurotransmitter release in an in vivo situation. To study the above questions, we have used the endothelin-1 model for focal cerebral ischaemia. Micro-injection of endothelin-1 in the proximity of the middle cerebral artery, can be used to produce focal cerebral ischaemia in freely moving rats, by which the middle cerebral artery can be reversibly occluded (Sharkey et al., 1993; Bogaert et al, 2000). The integration of reperfusion in this model offers the opportunity to mimic the clinical situation best, since vessel occlusion is not permanent in humans. Moreover, reperfusion has been demonstrated to cause additional injury to the ischaemic tissue. The oxygen supplied by the re-established blood circulation, although necessary for alleviating the ischaemic status, can enhance the production of free radicals and provokes additional injury in the already damaged tissue.

We have investigated the effects of nimodipine (known to be hemodynamically active) and verapamil, both L-type voltage sensitive Ca^{2+} channel (VSCC) blockers. Their effects were compared with LY393613, a novel neuronal (N/P/Q-type) VSCC blocker on (1) the striatal extracellular levels of dopamine and Glu, on (2) the striatal blood flow time profiles and on (3) the histological outcome, using the endothelin-1 model for focal ischaemia.

2. Materials and methods

2.1. Chemicals and reagents

Glu and dopamine, as well as endothelin-1 (endothelin-1, human, porcine sequence; synthetic) were supplied by Sigma (St. Louis, MO, USA). Verapamil and nimodipine were purchased from Aldrich (Sigma Aldrich, Bornem, Belgium) and Acros (Acros Organics, Geel, Belgium), respectively. LY393613 was obtained from Eli Lilly (Eli Lilly&Co. Ltd., UK). All other chemicals were analytical reagent grade or better and supplied by Merck (Darmstadt, Germany). Aqueous solutions were made using fresh water purified with a Seralpur pro-90 CN (Belgolabo, Overijse, Belgium), and filtered through a 0.2-μm membrane filter. As perfusion fluid for the microdialysis experiments, a Ringer's solution was used containing 147 mM NaCl, 1.1 mM $CaCl_2$ and 4 mM KCl.). Endothelin-1 was solved in the above-mentioned Ringer's solution. The anti-oxidant mixture to prevent degradation of the sampled dopamine consisted of 0.02 M HCl, 0.2% sodium metabisulphite and 0.02% Na_2EDTA.

2.2. Microdialysis experiments

The protocols for the animal experiments described in this study were performed according to the European Communities Council Directive (86/609/EEC), and were approved by the Ethics Committee on Animal Experiments of the Faculty of Medicine and Pharmacy of the Vrije Universiteit Brussel.

Male albino Wistar rats, weighing 250–280 g, were anaesthetized with a mixture of ketamine (50 mg/kg) and diazepam (5 mg/kg) and mounted on a stereotaxic frame. The skull was locally anaesthetized with a subcutaneous injection of 50 μl 0.1% lidocaine. A microdialysis probe (CMA 12, 3 mm, CMA Microdialysis, Stockholm, Sweden) was implanted in the striatum and a catheter for the microinjection of endothelin-1 was introduced in the proximity of the middle cerebral artery. The coordinates towards bregma were L: +2.4, A: +1.2, V: +2.8, for the probe in the striatum, and L: +5.6, A: −0.3, V: +7.0, for the catheter in the proximity of the middle cerebral artery (Paxinos and Watson, 1986). Immediately after surgery, the animals received an intraperitoneal injection of the analgesic ketofen (4 mg/kg). The animals were allowed to recover from the surgery overnight and had free access to water and food. The probe in the striatum was continuously perfused with Ringer's solution at a flow rate of 2

μl/min using a CMA/100 microdialysis pump (CMA Microdialysis, Stockholm, Sweden).

2.3. Experimental procedure

The day after the surgery, sampling was started from the striatum of freely moving rats. Dialysates were collected for 20 min in vials. Dialysates (40 μl) were split for the analysis of dopamine (25 μl dialysate + 10 μl of the above described anti-oxidant mixture) and Glu (15 μl dialysate).

The experimental groups were as follows.

2.3.1. Group 1

Control group: After having reached baseline values, endothelin-1 was injected near the middle cerebral artery at a flowrate of 1 μl/min for 6 min (120 pmol endothelin-1) ($n = 20$).

2.3.2. Group 2

Drug-alone group: This group contains three sets of experiments.

Set 1: rats receiving an intraperitoneal injection of LY393613 (15 mg/kg), 30 min before the Ringer's injection in the proximity of the middle cerebral artery ($n = 4$); set 2: rats receiving an intraperitoneal injection of nimodipine (0.10 mg/kg), 30 min before the Ringer's injection in the proximity of the middle cerebral artery ($n = 4$); and set 3: rats receiving an intraperitoneal injection of verapamil (0.40 mg/kg), 30 min before the Ringer's injection in the proximity of the middle cerebral artery ($n = 4$).

2.3.3. Group 3

Again, this group is divided in three sets of experiments.

Set 1: rats receiving an intraperitoneal pre-injection of LY393613 (15 mg/kg, $n = 6$), 30 min before the endothelin-1 injection; set 2: rats receiving an intraperitoneal pre-injection of nimodipine (0.10 mg/kg, $n = 6$), 30 min before the endothelin-1 injection; and set 3: rats receiving an intraperitoneal pre-injection of verapamil (0.40 mg/kg, $n = 6$), 30 min before the endothelin-1 injection.

2.3.4. Group 4, 5

Both groups 4 and 5 are divided in three sets of experiments.

Set 1: experiments consisting of an intraperitoneal post-injection of LY393613 (15 mg/kg, $n = 5$), either 30 min after the endothelin-1 injection (group 4), or 60 min after the endothelin-1 injection (group 5); set 2: rats receiving an intraperitoneal post-injection of nimodipine (0.10 mg/kg, $n = 5$), either 30 min (group 4), or 60 min (group 5) after the endothelin-1 injection; and set 3: rats receiving an intraperitoneal post-injection of verapamil (0.40 mg/kg, $n = 5$), 30 min after the endothelin-1 injection (group 4), or 60 min after the endothelin-1 injection (group 5).

2.3.5. Group 6

Again, this group is divided in three sets of experiments.

Set 1: rats subjected to a local administration of LY393613 (1.5 mg/kg) near the middle cerebral artery, simultaneously with the endothelin-1 injection ($n = 3$); set 2: rats subjected to a local administration of nimodipine (10 μg/kg) near the middle cerebral artery, simultaneously with the endothelin-1 injection ($n = 3$); and set 3: rats subjected to a local administration of verapamil (40 μg/kg) near the middle cerebral artery, simultaneously with the endothelin-1 injection ($n = 3$).

After the insult, we collected dialysates for 3 h. At the end of these microdialysis experiments, the rats were sacrificed with an overdose of pentobarbital.

2.4. Chromatographic assays

Glu analysis was carried out after precolumn derivatization with *o*-phtalaldehyde/β-mercaptoethanol by reversed phase microbore liquid chromatography with gradient elution and fluorescence detection (Smolders et al., 1995). Dopamine was analyzed using an isocratic reversed-phase ionpair microbore liquid chromatography method with electrochemical detection (Smolders et al., 1996).

2.5. Laser Doppler flowmetry

Laser Doppler flowmetry was done to verify the local "striatal" blood flow. The stereotaxic placement of the cannula in the proximity of the middle cerebral artery, and the laser probe in the striatum were done under the same conditions as described above. In these experiments, rats were kept under anaesthesia with a mixture of ketamine/diazepam (50:5 mg/kg).

Intraperitoneal injections of LY393613 ($n = 4$), nimodipine ($n = 4$), and verapamil ($n = 4$), were performed either 30 min before, or 30 min after the endothelin-1 injection. In anaesthetized rats, a fourfold higher dose of endothelin-1 was required to obtain an ischaemic insult comparable to that in freely moving rats (Laureys et al., 1999; Bogaert et al., 2000).

2.6. Histology

The same surgical procedure was followed as described for the microdialysis groups. However, no probe was implanted in the striatum, hence, avoiding striatal damage. This allows an objective measurement of the ischaemic damage. Sections of 100-μm thickness were made using a vibratome (MA752 motorized Advance Vibroslice, Campden Instruments, UK). The sections were stained with

cresylviolet and were examined by light microscopy (Zeiss, Stemi 2000-C, Zeiss Belgium). The images of these sections were digitalized with a camera (Sony DXC, Sony Belgium), connected to the microscope and a computer. A computer program (NIH, Image 1.41) quantified the damaged zone on the digital images. Every 600 μm, the damage was measured and multiplied with the interspace. The sum of these data results in a total volume of damage, expressed as cubic millimeter. Someone without knowledge of the protocol did these measurements.

2.7. Data presentation and statistical analysis

The results of the microdialysis experiments, shown in the figures, are the mean dialysate concentrations (in percentage of the baseline level) (mean ± S.E.M.). The basal

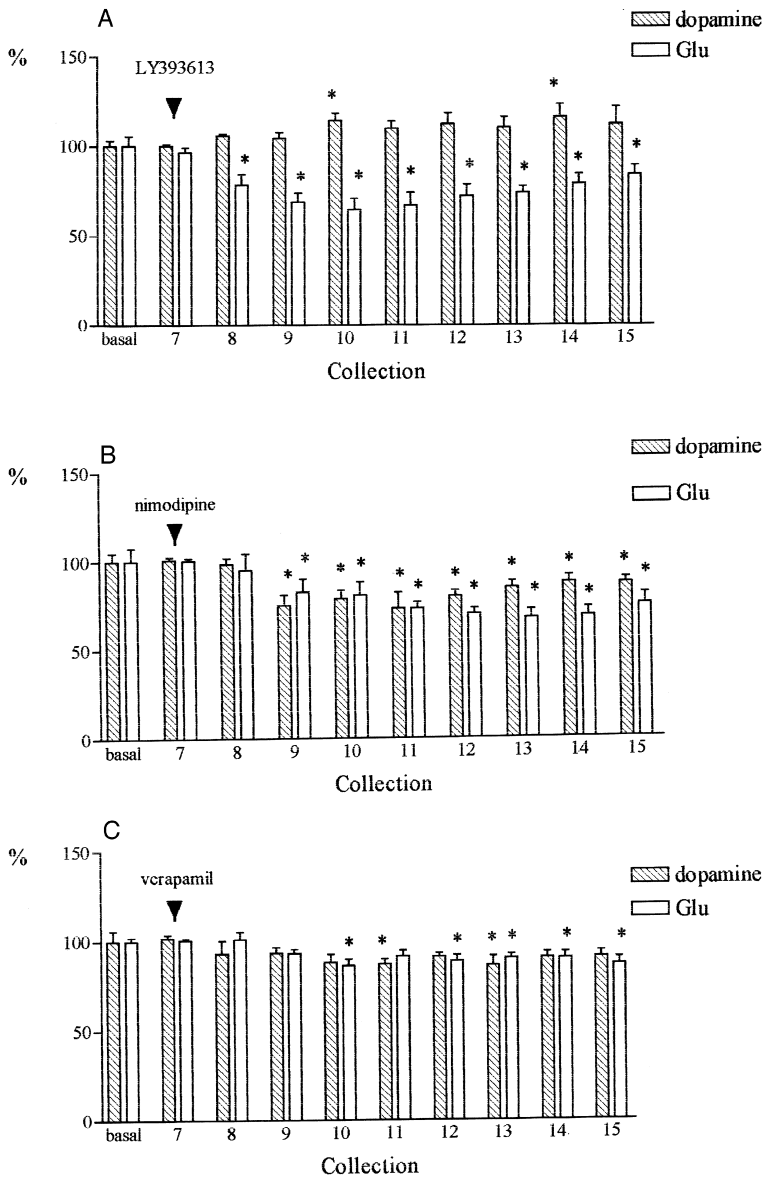

Fig. 1. (A) The effect of a systemic application (i.p.) of 15 mg/kg LY393613 on extracellular dopamine and Glu levels in the striatum of freely moving rats. LY393613 was administered in the beginning of collection period 7. Results are expressed as percentage (mean ± S.E.M., $n = 4$). All dialysates were sampled under perfusion with Ringers' solution. Each bar represents a 20-min collection period. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD post hoc tests. Asterisks denote values, significantly different from the baseline value ($^{*}\alpha = 0.05$). (B) The effect of a systemic application (i.p.) of 0.1 mg/kg nimodipine on extracellular dopamine and Glu levels in the striatum of freely moving rats. Nimodipine was administered in the beginning of collection period 7. Results are expressed as percentage (mean ± S.E.M., $n = 4$). All dialysates were sampled under perfusion with Ringers' solution. Each bar represents a 20-min collection period. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD post hoc tests. Asterisks denote values, significantly different from the baseline value ($^{*}\alpha = 0.05$). (C) The effect of a systemic application (i.p.) of 0.4 mg/kg verapamil, on extracellular dopamine and Glu levels in the striatum of freely moving rats. Verapamil was administered in the beginning of collection period 7. Results are expressed as percentage (mean ± S.E.M., $n = 4$). All dialysates were sampled under perfusion with Ringers' solution. Each bar represents a 20-min collection period. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD post hoc tests. Asterisks denote values, significantly different from the baseline value ($^{*}\alpha = 0.05$).

value in the figures is the mean of six stable neurotransmitter dialysate concentrations, obtained in basal conditions, i.e. prior to endothelin-1 or drug administration. Dialysate concentrations were not corrected for the recovery across the dialysis membrane. Statistical analysis of the changes of dopamine and Glu dialysate concentrations in time was performed using one-way analysis of variance (ANOVA) for repeated measures, and Fisher's protected least significance difference (Fisher's PLSD) post hoc tests ($\alpha = 0.05$). The same statistical analysis was used to evaluate the laser Doppler experiments, in which the blood flow was expressed as percentage of the baseline level (mean ± S.E.M.).

The significance of differences between peak dialysate concentrations was determined by the Student's *t*-test (two-tailed, $P < 0.05$). The significance of differences between the histology data, as well as the significance of difference between the data obtained using laser Doppler flowmetry, was also determined by the Student's *t*-test (two-tailed, $P < 0.05$).

3. Results

3.1. Microdialysis

Basal striatal levels (mean ± S.E.M.) ($n = 20$) in freely moving rats were 2.20 ± 0.15 nM for dopamine, and 0.64 ± 0.03 μM for Glu.

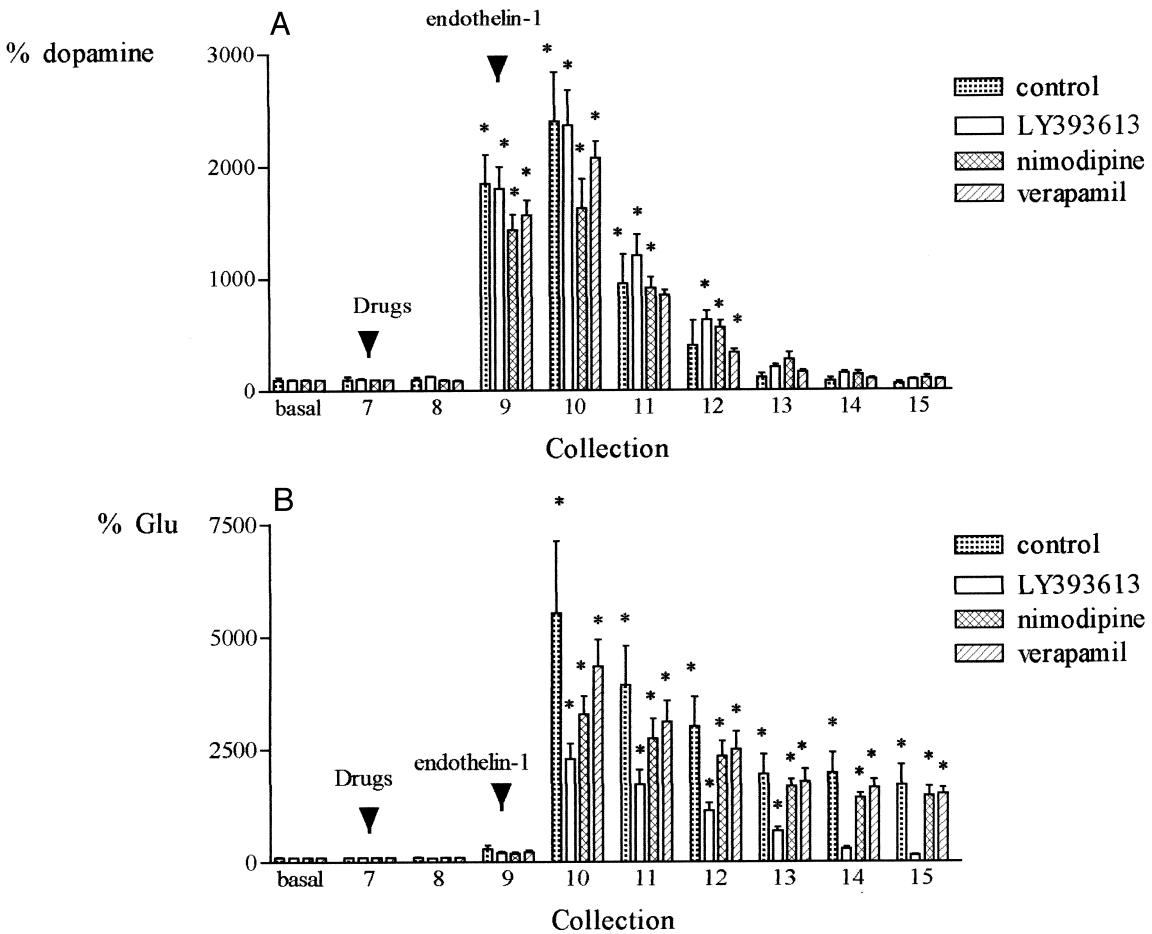

Fig. 2. (A) The effect of pretreatment with 15 mg/kg LY393613, 0.1 mg/kg nimodipine or 0.4 mg/kg verapamil (i.p.) on ischaemia-induced extracellular dopamine levels in the striatum of freely moving rats, subjected to a focal cerebral insult by injecting 120-pmol endothelin-1 near the middle cerebral artery. The drugs were injected half an hour before the endothelin-1 injection (endothelin-1 was injected in the beginning of collection period 9). Results are expressed as percentage (mean ± S.E.M.), $n = 6$ for the treated animals and $n = 20$, for the control experiments. All dialysates were sampled under perfusion with Ringers' solution. Each bar represents a 20-min collection period. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD post hoc tests. Asterisks denote values, significantly different from the baseline value ($^*\alpha = 0.05$). (B) The effect of a pretreatment with 15 mg/kg LY393613, 0.1 mg/kg nimodipine or 0.4 mg/kg verapamil (i.p.) on ischaemia-induced extracellular Glu levels in the striatum of freely moving rats, subjected to a focal cerebral insult by injecting 120-pmol endothelin-1 near the middle cerebral artery. The drugs were injected half an hour before the endothelin-1 injection (endothelin-1 was injected in the beginning of collection period 9). Results are expressed as percentage (mean ± S.E.M., $n = 6$). For the control experiments, $n = 20$. All dialysates were sampled under perfusion with Ringers' solution. Each bar represents a 20-min collection period. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD post hoc tests. Asterisks denote values, significantly different from the baseline value ($^*\alpha = 0.05$).

3.1.1. Group 1

Administration of 120 pmol endothelin-1, in the proximity of the middle cerebral artery, in freely moving rats, increased the extracellular striatal dopamine and Glu levels (mean ± S.E.M.) ($n = 20$) significantly to a maximum of 2412 ± 373% and 5508 ± 1398%, respectively. Extracellular striatal Glu levels remained significantly elevated until the end of the experiment, while the extracellular dopamine levels returned to baseline in collection 12.

3.1.2. Group 2

Drug-alone group: The intraperitoneal injection of LY393613 (15 mg/kg) ($n = 4$) (set 1) resulted in a significant decrease of the striatal Glu release, remaining till the end of the experiment. A maximum decrease of 36%, compared to the basal value was observed. Dopamine release was affected significantly in only two collection periods, i.e. collection period 10 and 14 (increased extracellular dopamine levels were 114% and 113%, respectively).

Intraperitoneal administration of nimodipine, (0.1 mg/kg) ($n = 4$) (set 2), resulted in a sustained and significant decrease of the extracellular dopamine levels (maximal decrease occurred in collection 11, i.e. to 73%, compared to the basal value). Glu levels were also decreased significantly till the end of the experiment with a maximum decrease of 33% in collection 13.

Intraperitoneal application of verapamil (0.4 mg/kg) ($n = 4$) (set 3) slightly, but significantly, affected the

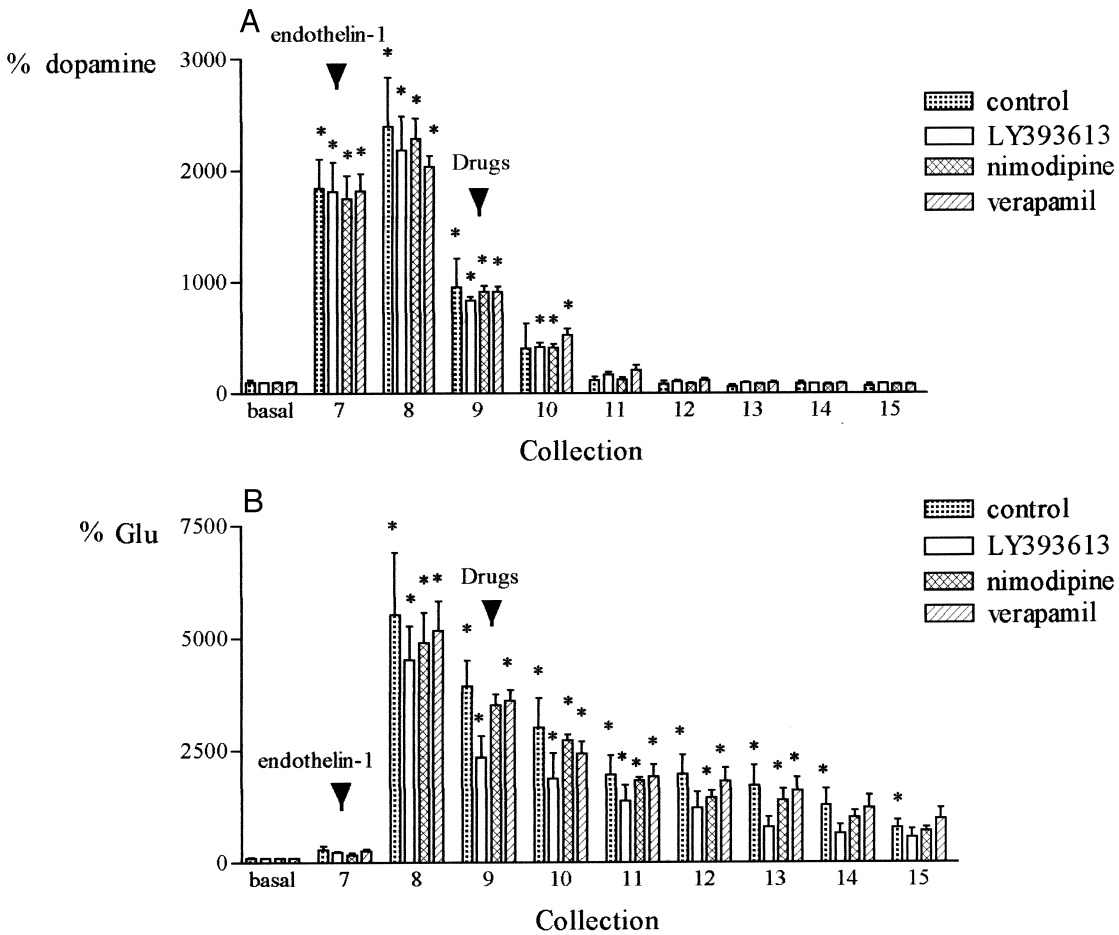

Fig. 3. (A) The effect of a post-treatment with 15 mg/kg LY393613, 0.1 mg/kg nimodipine, or 0.4 mg/kg verapamil (i.p.) on ischaemia-induced extracellular dopamine levels in the striatum of freely moving rats, subjected to a focal cerebral insult by injecting 120-pmol endothelin-1 in the proximity of the middle cerebral artery. The drugs were injected half an hour after the endothelin-1 injection (endothelin-1 was injected in the midst of collection period 7). Results are expressed as percentage (mean ± S.E.M., $n = 5$). For the control experiments, $n = 20$. All dialysates were sampled under perfusion with Ringers' solution. Each bar represents a 20-min collection period. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD post hoc tests. Asterisks denote values, significantly different from the baseline value ($^*\alpha = 0.05$). (B) The effect of a post-treatment with 15 mg/kg LY393613, 0.10 mg/kg nimodipine, or 0.40 mg/kg verapamil (i.p.) on ischaemia-induced extracellular Glu levels in the striatum of freely moving rats, subjected to a focal cerebral insult by injecting 120-pmol endothelin-1 in the proximity of the middle cerebral artery. The drugs were injected half an hour after the endothelin-1 injection (endothelin-1 was injected in the midst of collection period 7). Results are expressed as percentage (mean ± S.E.M., $n = 5$). For the control experiments, $n = 20$. All dialysates were sampled under perfusion with Ringers' solution. Each bar represents a 20-min collection period. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD post hoc tests. Asterisks denote values, significantly different from the baseline value ($^*\alpha = 0.05$).

transmitter releases. Dopamine levels decreased to 85% in collection 13, whereas the minimum of extracellular striatal Glu (i.e. 86% compared to the basal value) occurred during collection period 10 (Fig. 1A,B,C).

3.1.3. Group 3

Intraperitoneal administration of LY393613 (15 mg/kg) ($n = 6$), 30 min prior to the insult, reduced the ischaemia-induced striatal Glu release significantly, but not the dopamine release, compared to the controls ($n = 20$) ($0.01 < P < 0.05$). A reduction of 60% was observed compared to the controls in collection 10. Moreover, the extracellular Glu levels returned towards baseline values, whereas in the control group, the extracellular striatal Glu levels remained elevated (i.e. 700% of the baseline value in the control experiments in collection 15, versus 142% of the baseline value in the LY393613 pre-treated animals) (i.e. set 1 of this group).

Intraperitoneal administration of nimodipine (0.1 mg/kg) ($n = 6$), 30 min prior to the insult reduced the ischaemia-induced Glu release (but not the dopamine release) significantly compared to the controls ($0.01 < P < 0.05$). A decrease in the ischaemia-induced striatal Glu release of 42%, compared to the controls was seen in collection 10 (i.e. set 2 of this group).

Verapamil (0.4 mg/kg) ($n = 6$) did not alter the ischaemia-induced dopamine nor Glu release significantly when administered intraperitoneally 30 min before the insult (i.e. set 3 of this group) (Fig. 2A,B).

3.1.4. Group 4, 5

LY393613, administered intraperitoneally, 30 min after the insult (15 mg/kg) ($n = 5$), produced a slight attenuation of the ischaemia-induced Glu release in the collection periods 9 to 13 after the drug administration, but this failed to reach significance. The ischaemia-induced dopamine

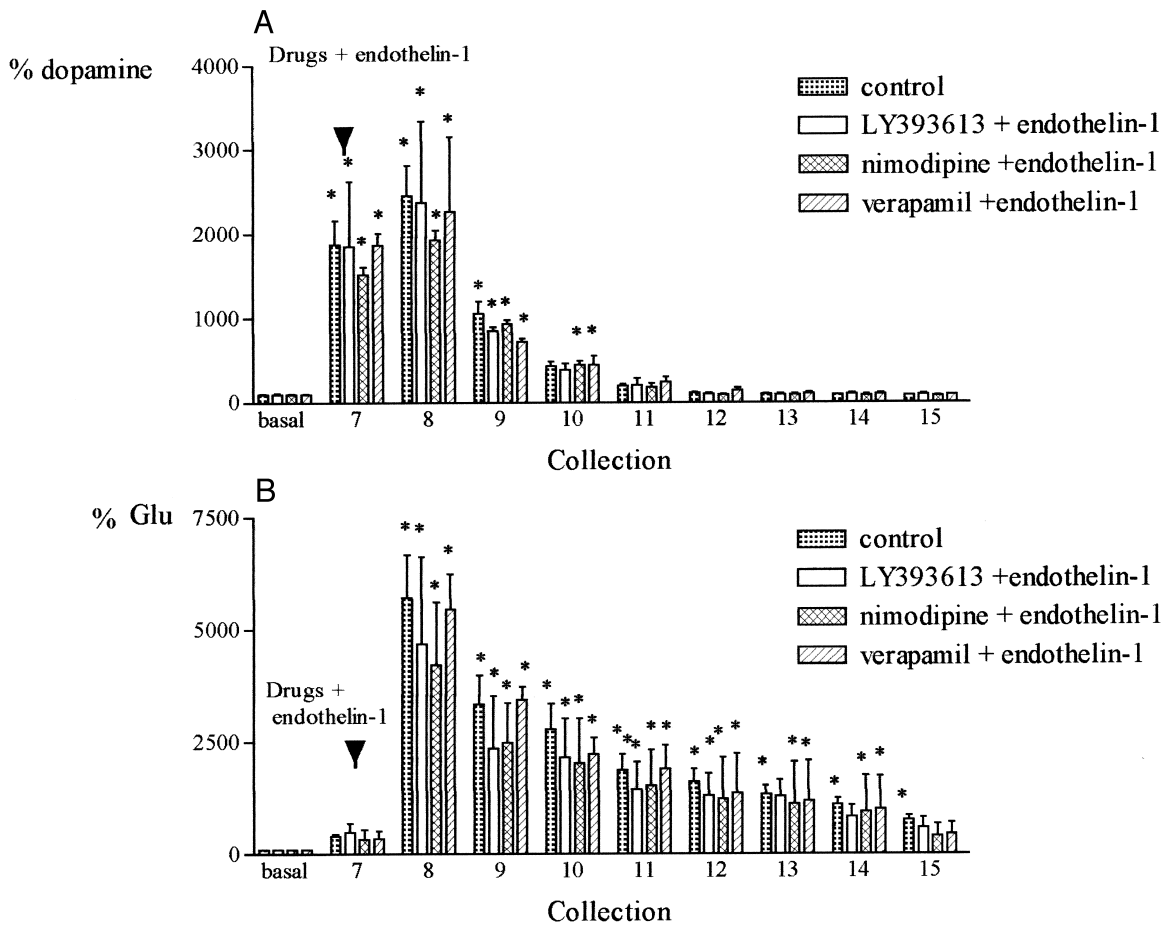

Fig. 4. (A) The effect on extracellular striatal dopamine levels of the administration of LY393613 (1.5 mg/kg), nimodipine (10 μg/kg), or verapamil (40 μg/kg), simultaneously with endothelin-1 in the proximity of the middle cerebral artery during collection period 7. Results are expressed as percentage (mean ± S.E.M., $n = 3$). For the control experiments, $n = 20$. All dialysates were sampled under perfusion with Ringers' solution. Each bar represents a 20-min collection period. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD post hoc tests. Asterisks denote values, significantly different from the baseline value ($^*\alpha = 0.05$). (B) The effect of the administration of LY393613 (1.5 mg/kg), nimodipine (10 μg/kg) or verapamil (40 μg/kg), simultaneously with endothelin-1 in the proximity of the middle cerebral artery during collection period 7. Results are expressed as percentage (mean ± S.E.M., $n = 3$). For the control experiments, $n = 20$. All dialysates were sampled under perfusion with Ringers' solution. Each bar represents a 20-min collection period. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD post hoc tests. Asterisks denote values, significantly different from the baseline value ($^*\alpha = 0.05$).

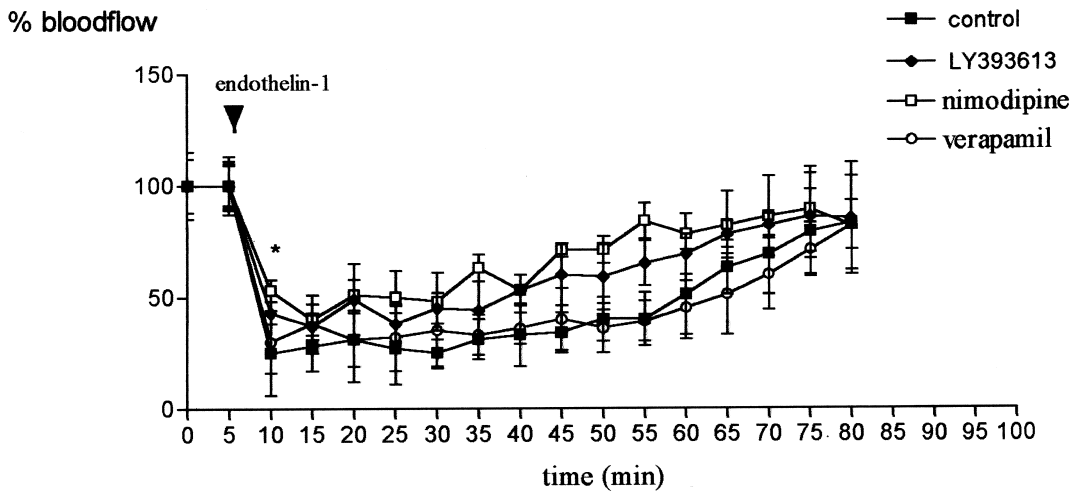

Fig. 5. Alterations in striatal cerebral blood flow after endothelin-1 injection near the middle cerebral artery. The administration of endothelin-1 occurred at 5 min. Drugs were injected intraperitoneally half an hour before the endothelin-1 injection. The values are expressed as percentage (mean ± S.E.M.), $n = 4$. Statistics: one-way ANOVA for repeated measures followed by Fisher's PLSD post hoc tests. Asterisks denote only the first value significantly different from the corresponding baseline value ($^{*}\alpha = 0.05$).

release was not affected significantly (i.e. set 1 of this group).

Injection of the Ca^{2+} channel blockers nimodipine (0.1 mg/kg) ($n = 5$) (i.e. set 2 of this group) and verapamil (0.4 mg/kg) ($n = 5$) (i.e. set 3 of this group) produced no significant changes in the ischaemia-induced releases of the neurotransmitters (dopamine and Glu) (Fig. 3A,B).

When the administration of the tested compounds was delayed up to 1 h after the insult, none of the transmitter releases monitored, showed any effect (data not shown).

3.1.5. Group 6

We also examined the direct effects of the Ca^{2+} channel blockers on the vasoconstrictive properties of endothelin-1. For this purpose, endothelin-1 was co-administered via the same catheter with LY393613 (1.5 mg/kg) ($n = 3$) (i.e. set 1 of this group), nimodipine (10 μg/kg) ($n = 3$) (i.e. set 2 of this group), and verapamil (40 μg/kg) ($n = 3$) (i.e. set 3 of this group). Neurotransmitter levels were monitored for a 2–3-h period. None of the tested drugs altered the ischaemia-induced increases in extracel-

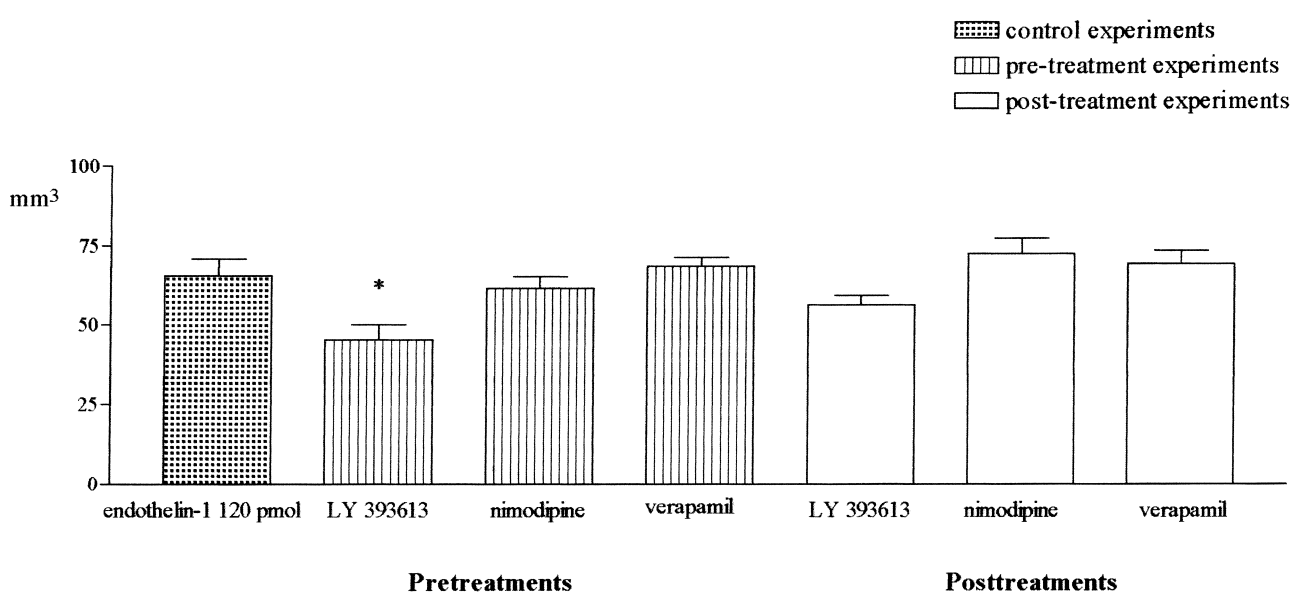

Fig. 6. Volume of ischaemic damage, 24 h after the insult, is expressed as cubic millimeter (mean ± S.E.M.). Micro-application of Et-1 (120 pmol), near the MCA ($n = 7$) was used for control. Pretreatment of the rats occurred 30 min before the insult. Animals were treated systemically (i.p.) with 15 mg/kg LY393613 ($n = 5$), with 0.10 mg/kg nimodipine ($n = 3$), or with verapamil 0.40 mg/kg), ($n = 3$). Post-treated animals had an i.p. injection of the drugs 30 min after the insult. The same doses as mentioned in the pre-treated group were used, $n = 3$ in the three groups. Asterisks denote values, significantly different from the control group ($^{*}P < 0.05$).

lular Glu or dopamine significantly (Fig. 4A,B). Therefore, the attenuation of the ischaemia-induced Glu levels, observed after the intraperitoneal administration of the Ca^{2+} channel blockers, was not directly due to the antagonism of the endothelin-1 vasoconstrictive properties.

3.2. Laser Doppler flowmetry

Laser Doppler flowmetry showed that the infusion of 500 pmol endothelin-1 near the middle cerebral artery produced a large decrease in striatal blood flow to approximately 30% of the baseline levels, lasting for about 40 min. Injections of the tested drugs 30-min beforehand resulted in an attenuation of decrease in striatal blood flow, although this failed to reach significance (Fig. 5). Post-insult administration of the drugs was also performed. None of the tested drugs attenuated the drop of the striatal blood flow (data not shown).

3.3. Histology

A micro-application of endothelin-1 (120 pmol) caused an infarct volume of 65 ± 6 mm^3 ($n = 7$). LY393613, administered at a dose of 15 mg/kg ($n = 5$) produced a significant reduction in the infarct size (44 ± 4 mm^3) when administered 30 min before the insult ($P = 0.05$). Post-insult administration of LY393613 attenuated but did not significantly change the infarct volume (56 ± 3 mm^3). Nimodipine (0.1 mg/kg) ($n = 3$) also tended to reduce the infarct size in the animals treated beforehand (59 ± 3 mm^3), but this was not significant. Verapamil (0.4 mg/kg) ($n = 3$) showed no significant changes in the total ischaemic damage neither in the pre-treated rats nor in the post-treated rats (Fig. 6).

4. Discussion

In the present studies, we have examined the effects of two L-type (nimodipine and verapamil) and a new neuronal, N/P/Q-type (LY393613), Ca^{2+} channel blocker in the endothelin-1 model for cerebral ischaemia. This model consists of a reversible middle cerebral artery occlusion, giving rise to infarction of both the cortex and striatum. Using microdialysis, we have demonstrated that LY393613 provided a significant decrease in the ischaemia-induced Glu release when administered pre-occlusion in this model. The effect on Glu of the post-occlusion administration of LY393613 failed to reach significance, probably due to a short therapeutic window of the drug. Consistently, histopathological findings showed no significant changes in the post-treated rats, although significance was reached in the pre-treated rats. Verapamil and nimodipine provided no significant changes of the infarct volume. For LY393613, the mechanism of protection appears to be due to the blockade of Glu release. Indeed, it was observed that the protective effect of LY393613 was not due to a direct counteraction of the endothelin-1 vasoconstrictive functionality, nor due to a significantly increased striatal blood flow.

Excessive release of neurotransmitters is known to play an important role in the development of ischaemic damage. It is well established that ischaemia causes a massive release of dopamine and Glu, leading to neuronal death, probably via an excitotoxic process (Abe et al., 1988; Globus et al., 1988). The major pathway of the excitotoxicity is believed to be the entry of extracellular Ca^{2+} into ischaemic neuronal cells. Ca^{2+} can enter cells via ligand-operated Ca^{2+} channels, ion-gated Ca^{2+} channels, or voltage-sensitive Ca^{2+} channels (VSCC). Initially, Ca^{2+} channels were classified into three types of VSCC, the dihydropyridine-sensitive L-channel and the dihydropyridine-insensitive N- and T-channels (Miller, 1987). More recently, P-, Q- and R-types have been added (for review, see Dunlap et al., 1995; Varadi et al., 1995). LY393613, is a novel neuronal Ca^{2+} channel blocker acting at N-, P- and Q-types of VSCC and both nimodipine and verapamil are L-type VSCC blockers.

4.1. Histological studies demonstrating neuroprotection

In the present studies, we have demonstrated that the neuronal (N/P/Q-type) Ca^{2+} channel antagonist, LY393613 provided a significant reduction in infarct volume when administered before occlusion. In contrast, nimodipine and verapamil provided no significant protection when administered before occlusion. However, many early studies reported that L-type Ca^{2+} channel antagonists such as (*S*)-emopamil, nimodipine and nicardipine have neuroprotective effects in models of cerebral ischaemia (Alps, 1992; Lin et al., 1990; Morikawa et al., 1991; Rami and Krieglstein, 1994). The effect of many of the earlier L-type Ca^{2+} channel antagonists on blood flow, blood pressure may have contributed to their neuroprotective actions.

It has been reported that ω-conotoxin GVIA, which blocks the N-type Ca^{2+} channels, and ω-agatoxin IVA, blocking the P/Q-type Ca^{2+} channels, delay neurotransmitter release in striatal slices, while in contrast nimodipine and nicardipine (L-type VSCC) do not (Toner and Stamford, 1997), suggesting that N- and P/Q-type VSCCs play a greater role than L-type VSCC in ischaemic injury in the striatum in vitro. Additional studies indicated that when rat hippocampal slices were subjected to oxygen-glucose deprivation, nimodipine (L-type) and daurisoline (P-type) provided less than 20% protection, ω-conotoxin MVIIA (N-type) provided 40% protection and 200 nM ω-agatoxin IVA (P + Q-type) provided 75% protection (Small et al., 1997). However, ω-conotoxin MVIIC (P + Q + N-type) provided complete protection, suggesting that all three neuronal Ca^{2+} channels contribute to ischaemic brain injury in the hippocampus.

Several other in vivo studies have reported that the ω-conotoxin GVIA is effective in reducing the infarct volume in rat models of focal cerebral ischaemia when administered during (Takizawa et al., 1995) or after the occlusion (Buchan et al., 1994; Yenari et al., 1996). It has also been demonstrated that i.c.v. administration of ω-agatoxin IVA, which blocks P/Q-type Ca^{2+} channels, protects against focal ischaemia in rats (Asakura et al., 1997). However, other investigators have demonstrated that another P-type Ca^{2+} channel antagonist, daurisoline (a non-peptide) failed to protect in a rat model of focal ischaemia (Lingenhöhl et al., 1997).

The conotoxins are large peptide molecules and do not cross the blood–brain barrier easily. For this reason, they have been administered centrally or i.v. at high doses, (affecting the blood pressure). Therefore, more recent efforts have focused on small molecule neuronal Ca^{2+} channel blockers. We have previously shown that many of these molecules (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 CNS1237 (*N*-acenaphthyl-*N*′-4-methoxynaphth-1-yl guanidine) provide minimal protection in a gerbil model of global cerebral ischaemia (O'Neill et al., 1997). We have also observed that LY393613 (15 mg/kg i.p.) provides the greatest neuroprotection than all of the above molecules in global ischaemia (O'Neill et al., unpublished results).

4.2. Laser Doppler flowmetry

Using laser Doppler flowmetry, we observed that all the drugs tested showed no significant effects on the striatal blood flow, either when administered before or after the insult. The laser Doppler technique used here measures relative blood flow only locally in the striatum. Therefore, in certain animals, there will be more reduction in blood flow in one specific region of the striatum than in the other. The absence of significant increases in striatal blood flow following administration of the drugs after the induction of the ischaemic insult in this study can be explained by the already maximal vasodilatation in the ischaemic brain tissue (Waltz and Sundt, 1967; Garcia, 1984) and by the greater inter-animal variation due to the very local measurement. Vasodilatation seems to be an active process in the very beginning of the ischaemic insult, but when ischaemia proceeds, it becomes more and more the result of vasomotor paralysis, as was observed by Lassen and Christensen, (1976) and by Garcia (1984). LY393613, nimodipine, and verapamil did not produce any significant increase in striatal blood flow. These results suggest that these compounds reduce ischaemic damage independently of effects on cerebral hemodynamics. This is in agreement with other studies using nimodipine (Dirnagl et al., 1990).

4.3. Microdialysis: effects on neurotransmitter release

4.3.1. Dopamine

In the present studies, we found that nimodipine and to a lesser extent verapamil, decreased dopamine levels in the striatum. Since intracellular levels of Ca^{2+} regulate the metabolism of dopamine, Ca^{2+} channel blockers will reduce the release. Pileblad and Carlsson, (1986) found that this was especially the case via L-type VSCC. It has also been demonstrated that nimodipine is able to attenuate the hyperactivity of dopaminergic neurons (Fadda et al., 1989) and VSCC blockers also can inhibit dopamine uptake. Devoto et al. (1991) reported inhibition of dopamine uptake by flunarizine, and other Ca^{2+} channel blockers. It has also been demonstrated that distinct VSCCs contribute to release of dopamine from mesencephalic cultures in vitro (Grilli et al., 1989) and further studies reported that nimodipine blocks BAYK 8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4[2-trifluoromethyl-phenyl]-pyridine-5-carboxylate) stimulated Ca^{2+} entry and dopamine release in striatal synaptosomes (Woodward and Leslie, 1986).

In contrast, Bowyer and Weiner (1990) showed that Ca^{2+} evoked dopamine release is dependent on N-type Ca^{2+} channels. Therefore, dihydropyridines or other compounds acting on L-type Ca^{2+} channels should not alter neurotransmitter release. This is in disagreement with our results. The reason for this is not clear so far. As was already discussed by Garrido et al. (1991), attention must be paid towards differences in expression and function of the different types of Ca^{2+} channels in healthy or diseased situations. Our results showed that pre-treatment with LY393613 did not affect the basal levels of dopamine significantly neither did nimodipine and verapamil. However, our results point towards a more prominent effect of L-type VSCC blockers, nimodipine and verapamil, compared to the N/P/Q-type VSCC blocker, LY393613, on the release profile of dopamine in intact animals. Injection of endothelin-1 produced a large increase (20–30-fold) in extracellular striatal levels of dopamine. The increase was greatest in the second collection fraction after endothelin-1 infusion. This ischaemia-induced increase in dopamine levels was not significantly affected by any of the Ca^{2+} channel blockers we tested.

4.3.2. Glutamate

In the present studies, LY393613 caused a decrease in basal Glu levels in normal animals. There was a massive (50-fold) increase in extracellular Glu levels in the striatum after infusion of endothelin-1. Several other investigators have demonstrated that there is a large increase in Glu after global (Benveniste et al., 1984; Globus et al., 1988) and focal (Butcher et al., 1990) cerebral ischaemia. It has also been reported that Glu can activate post-synaptic excitatory amino acid receptors to allow Ca^{2+} influx. The excessive entry of Ca^{2+} is also thought to be the major cause of Glu toxicity in nerve cells. The mechanism

pointing towards Glu excitotoxicity is still not fully understood but the loss of Ca^{2+} homeostasis probably plays an important role (Choi and Harley, 1993). The ischaemia-induced increase in Glu release, increases intracellular Ca^{2+}, which activates a large number of Ca^{2+}-dependent processes and causes neuronal damage, both of which promote further increase in Glu release (Choi 1988; Louzada et al., 1992; Osborne and Herrera, 1994) and decrease Glu uptake (Yu et al., 1987). Therefore, the excitotoxic insult is amplified by the continuous stimulation of the Glu receptors, promoting the spread of excitotoxicity to other neurons. The Ca^{2+} influx through the Glu receptor-associated channels seems to be the most important route of Ca^{2+}, when compared with the influx of Ca^{2+} via the VSCC (Ferreira et al., 1996). In agreement with this, several studies demonstrated neuroprotective effects with excitatory amino acid antagonists (see McCulloch, 1992).

In the present studies, we found that treatment with LY393613 was more effective than nimodipine or verapamil at attenuating the ischaemia-induced increases in Glu. These results indicate that N/P/Q-type Ca^{2+} channels play a critical role in ischaemia-induced Glu release. These results are also in agreement with several other studies that have demonstrated that selective blockade of neuronal Ca^{2+} channels is neuroprotective. As mentioned above, studies performed with ω-conotoxin GVIA (SNX-111) showed an effective reduction of the neocortical infarct volume in rat models of focal ischaemia, when administered during the occlusion (Takizawa et al., 1995) and after the ischaemic episode (Buchan et al., 1994). Takizawa et al. (1995) also demonstrated that SNX-111 reduced ischaemia-induced Glu release. A study by Small et al. (1997) used various combinations of Ca^{2+} channel blockers to elucidate, which Ca^{2+} channel types contribute to hypoxia-ischaemic damage in hippocampal slices in vitro. Their results indicated that the rank order of protection was MVIIC (P + Q + N-type) > ω-agatoxin IVA (P + Q-type) > ω-conotoxin MVIIA (N-type) > nimodipine (L-type) and daurisoline (P-type). Therefore, all three (N/P/Q) neuronal Ca^{2+} channels contribute to ischaemic brain injury in the hippocampus.

As we were using a vasoconstrictor to induce cerebral ischaemia, we carried out a final set of experiments to test the effects of the Ca^{2+} channel blockers on the vasoconstrictive properties of endothelin-1. For that aim, endothelin-1 and the Ca^{2+} channel antagonists were injected simultaneously, at the same location, near the middle cerebral artery. The results indicated that the ischaemia-induced increases in Glu and dopamine levels were not affected by any of the Ca^{2+} channel blockers. Therefore, the contractile effects of endothelin-1 are not due solely to an action on voltage-dependent Ca^{2+} channels. These results are in congruence with another study by de Aguilera et al. (1990), reporting the removal of extracellular Ca^{2+} or the addition of nicardipine attenuated but did not abolish the contractile responses to endothelin-1.

4.4. Conclusion

In conclusion, our results indicate that LY393613 attenuates ischaemia-induced increases in extracellular Glu levels and reduces infarct volume in this model of focal cerebral ischaemia. These effects are not due to the direct blockade of the vasoconstrictive actions of endothelin-1, nor to alterations of striatal blood flow. In contrast, nimodipine and verapamil failed to provide protection when administered after the occlusion. Therefore, N/P/Q-type, and not L-type Ca^{2+} channels, play a more relevant role in ischaemic brain damage. LY393613, is a novel small molecule, Ca^{2+} channel blocker, with low molecular weight, may be useful as an anti-ischaemic agent.

Acknowledgements

We are grateful to Mrs. C. De Rijck, Mrs. R. Berckmans, Mr. G. De Smet and Mrs. R.M. Geens for their technical assistance. This research is financed by a grant from the Flemish Institute for promotion of Scientific and Technological Research in the Industry (IWT), grant no. 951042. I. Smolders and S. Sarre are post-doctoral research fellows of the Fund for Scientific Research Flanders (FWO, Flanders). We thank the Koningin Elisabeth Stichting voor Geneeskundig Wetenschappelijk Onderzoek and the Fund for Scientific Research for their financial support. We are also indebted to Janssen Pharmaceutica, Neuss, Germany for the use of the laser Doppler Flowmeter.

References

Abe, K., Kogure, T., Watanabe, T., 1988. Prevention of ischaemic and post-ischaemic brain edema by a novel calcium antagonist (PN200-110). J. Cereb. Blood Flow Metab. 8, 436–439.

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

Asakura, K., Matsuo, Y., Kanemusa, T., Ninomiya, M., 1997. P/Q-type Ca^{2+} channel blocker ω-conotoxin IVA protects against brain injury after focal ischaemia in rats. Brain Res. 776, 140–145.

Benveniste, H., Drejer, J., Schousboe, A., Diemer, N.H., 1984. Elevation of the extracellular concentrations of Glu and aspartate in rat hippocampus during transient cerebral ischaemia monitored by intracerebral microdialysis. J. Neurochem. 43, 1369–1374.

Bogaert, L., Scheller, D., Moonen, J., Sarre, S., Smolders, I., Ebinger, G., Michotte, Y., 2000. Neurochemical changes and laser Doppler flowmetry in the endothelin-1 rat model for focal cerebral ischemia. Brain Res. In press.

Bowyer, J.F., Weiner, N., 1990. Calcium evoked 3OH_DA release from synaptosomes is dependent on neuronal type calcium channels and is not mediated by acetylcholine, Glu or aspartate release. J. Neuropharmacol. Exp. Ther. 254, 664–670.

Buchan, A.M., Gertler, S.Z., Li, H., Xue, D., Huang, Z.G., Chaundy, K.E., Barnes, K., Lesiuk, H.J., 1994. A selective N-type Ca^{2+}-channel blocker prevents CA1 injury 24 hours following severe forebrain ischaemia and reduces infarction following focal ischaemia. J. Cereb. Blood Flow Metab. 14, 903–910.

Butcher, S.P., Bullock, R., Graham, D.I., McCulloch, J., 1990. Correlation between amino acid release and neuropathologic outcome in rat

brain following middle cerebral artery occlusion. Stroke 21, 1727–1733.

Choi, D.W., 1988. Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischaemic damage. Trends Neurosci. 11, 465–469.

Choi, D.W., 1992. Excitotoxic cell death. J. Neurobiol. 23, 1261–1276.

Choi, D.W., Harley, D.M., 1993. Calcium and Glu-induced cortical neuronal death. In: Waxman, S.G. (Ed.), Molecular and Cellular Approaches in the Treatment of Neurological Disease. Raven Press, New York, NY, 23.

Clapham, D.E., 1995. Calcium signaling. Cell 80, 259–268.

de Aguilera, E.M., Irurzun, A., Vila, J.M., Aldasoro, M., Galeote, M.S., Lluch, S., 1990. Role of endothelium and calcium channels in endothelin-induced contraction of human cerebral arteries. Br. J. Clin. Pharmacol. 99, 439–440.

Devoto, P., Pani, L., Kuzmin, A., De Montis, G., 1991. Inhibition of 3H-DA uptake by flunarizine. Eur. J. Pharmacol. 203, 67–69.

Dirnagl, U., Jacewicz, M., Pulsinelli, W., 1990. Nimodipine post-treatment does not increase blood flow in rats with focal cortical ischaemia. Stroke 21, 1357–1361.

Dunlap, K., Luebke, J.L., Turner, T.J., 1995. Exocytotic calcium channels in mammalian central neurons. Trends Neurosci. 18, 89–98.

Fadda, F., Gessa, G.L., Mosca, E., Stefanini, E., 1989. Different effects of the calcium antagonists nimodipine and flunarizine on DA metabolism in the rat brain. J. Neural Transm. 75, 195–200.

Ferreira, I.L., Duarte, C.B., Carvalho, A.P., 1996. Calcium influx through Glu receptor-associated channels in retina cells correlates with neuronal cell death. Eur. J. Pharmacol. 302, 153–162.

Garcia, J.H., 1984. Experimental ischaemic stroke: a review. Stroke 15, 5–14.

Garrido, B., Abad, F., Garcia, A.G., 1991. Modulation by voltage of calcium channels and adrenal catecholamine release. Ann. N. Y. Acad. Sci. 635, 459–463.

Globus, M.Y.-T., Busto, R., Dietrich, W.D., Martinez, E., Valdés, I., Ginsberg, M.D., 1988. Effect of ischaemia on the in vivo release of striatal DA, Glu and gamma-aminobutyric acid studied by intracerebral microdialysis. J. Neurochem. 51, 1455–1464.

Gomi, S., Greenburg, J.H., Croul, S., Revich, M., 1995. Failure of levemopamil to improve histological outcome following temporary occlusion of the middle cerebral artery in cats. J. Neurol. Sci. 130, 128–133.

Grilli, M., Wright, A., Haubauer, I., 1989. Evidence for the involvement of distinct voltage-sensitive calcium channels in the release of 3OH_DA from primary cultures of mesencephalic neurons. Neuropharmacology 11, 1275–1278.

Lassen, N.A., Christensen, M.S., 1976. Physiology of cerebral blood flow. Br. J. Anaesth. 48, 719–734.

Laureys, S., Bogaert, L., Coremans, J., Sarre, S., Michotte, Y., Ebinger, G., 1999. In vivo microdialysis and diffusion magnetic resonance imaging in a rat model of transient focal ischaemia. J. Cereb. Blood Flow Metab. 19 (Suppl. 1), 495.

Lin, B., Dietrich, W.D., Busto, R., Ginsberg, M.D., 1990. (*S*)-Emopamil protects against global brain injury in rats. Stroke 21, 1734–1739.

Lingenhöhl, K., Small, D.L., Monette, R., Buchan, A.M., Morley, P., Allegrini, P.R., Frostl, W., Sauer, D., Schmutz, M., Knöpfel, T., 1997. Exploration of P-type Ca^{2+} channels as drug targets for the treatment of epilepsy or ischaemic stroke. Neuropharmacology 36, 107–113.

Louzada, P. Jr., Dias, J.J., Santos, W.F., Lachat, J.J., Bradford, H.F., Coutinho-Neto, J., 1992. Glu release in experimental ischaemia of the retina: an approach using microdialysis. J. Neurochem. 59, 358–363.

McCulloch, J., 1992. Excitatory amino acid antagonists and their potential for the treatment of ischaemic brain damage in man. Br. J. Clin. Pharmacol. 34, 106–114.

Miller, R.J., 1987. Multiple calcium channels and neuronal function. Science 235, 46–52.

Morikawa, E., Ginsberg, M.D., Dietrich, W.D., Duncan, R.C., Busto, R., 1991. Postischaemic (*S*)-emopamil therapy ameliorates focal ischaemic brain injury in rats. Stroke 22, 355–360.

Nicotera, P., Bellome, G., Orrenius, S., 1992. Calcium-mediated mechanisms in chemically induced cell death. Annu. Rev. Pharmacol. Toxicol. 32, 449–470.

Nitatori, T., Sato, N., Waguri, S., Karasawa, Y., Araki, H., Shibanai, K., Kominami, E., Uchiyama, Y., 1995. Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischaemia is apoptosis. J. Neurosci. 15, 1001–1011.

O'Neill, M.J., Bath, C.P., Dell, C.P., Hicks, C.A., Gilmore, J., Ambler, S.J., Ward, M.A., Bleakman, D., 1997. Effects of Ca^{2+} and Na^{+} channel inhibitors in vitro and in global cerebral ischaemia in vivo. Eur. J. Pharmacol. 332, 121–131.

Osborne, N.N., Herrera, A.J., 1994. The effect of experimental ischaemia and excitatory amino acid agonists on the GABA and serotonin immunoreactivities in the rabbit retina. Neuroscience 59, 1071–1081.

Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic Coordinates. 2nd edn. Academic Press, San Diego.

Pileblad, E., Carlsson, A., 1986. In vivo effects of the calcium antagonist nimodipine on DA metabolism in mouse brain. J. Neurotrauma 66, 171–188.

Rami, A., Krieglstein, J., 1994. Neuronal protective effects of calcium channel antagonists in cerebral ischaemia. Life Sci. 55, 2105–2113.

Sharkey, J., Ritchie, I.M., Kelly, P.A.T., 1993. Perivascular microapplication of endothelin-1: a new model of focal cerebral ischaemia in the rat. J. Cereb. Blood Flow Metab. 13, 865–871.

Siesjö, B.K., 1992a. Pathophysiology and treatment of focal cerebral ischaemia: Part I. Pathophysiology. J. Neurosurg. 77, 337–354.

Siesjö, B.K., 1992b. Pathophysiology and treatment of focal cerebral ischaemia: Part II. Mechanisms and drug treatment. J. Neurosurg. 77, 1247–1252.

Small, D.L., Monette, R., Buchan, A.M., Morley, P., 1997. Identification of calcium channels involved in neuronal injury in rat hippocampal slices subjected to oxygen and glucose deprivation. Brain Res. 753, 209–218.

Smith, M.L., Siesjö, B.K., 1992. Postischaemic treatment with the omega-conopeptide SNX-111 protects the rat brain against ischaemic damage. In: Krieglstein, J. (Ed.), Pharmacology of Cerebral Ischaemia. pp. 161–166.

Smolders, I., Sarre, S., Ebinger, G., Michotte, Y., 1995. The analysis of excitatory, inhibitory and other amino acids in rat brain microdialysates using microbore liquid chromatography. J. Neurosci. Methods 57, 47–53.

Smolders, I., Sarre, S., Vanhaesendonck, C., Ebinger, G., Michotte, Y., 1996. Extracellular striatal DA and Glu after decortication and kainate receptor stimulation, as measured by microdialysis. J. Neurochem. 66, 2373–2380.

Takizawa, S., Matsushima, K., Fujita, H., Nanri, K., Ogawa, S., Shinohara, Y., 1995. A selective N-type calcium channel antagonist reduces extracellular Glu release and infarct volume in focal cerebral ischaemia. J. Cereb. Blood Flow Metab. 15, 611–618.

Toner, C.C., Stamford, J.A., 1997. Involvement of N- and P/Q but not L- ot T-type voltage-gated calcium channels in ischaemia-induced striatal DA release in vitro. Brain Res. 748, 85–92.

Valentino, K., Newcomb, R., Gadbois, T., Singh, T., Bowersox, S., Bitner, S., Justice, A., Yamashiro, D., Hoffman, B.B., Ciaranello, R., Miljanich, G., Ramachandran, J., 1993. A selective N-type calcium channel antagonist protects against neuronal loss after global cerebral ischaemia. Proc. Natl. Acad. Sci. U.S.A. 90, 7897.

Varadi, G., Mori, Y., Schwartz, A., 1995. Molecular determinants of calcium channel function and drug action. Trends Pharmacol. Sci. 16, 43–49.

Waltz, A.G., Sundt, T.M. Jr., 1967. The microvasculature and microcirculation of the cerebral cortex after arterial occlusion. Brain 90, 681–696.

Woodward, J.J., Leslie, S.W., 1986. BAY K 8644 stimulation of calcium entry and endogenous DA release in rat striatal synaptosomes antagonized by nimodipine. Brain Res. 370, 397–400.

Yenari, M.A., Palmer, J.T., Sun, G.H., de Crespigny, A., Moseley, M.E., Steinberg, G.K., 1996. Time-course and treatment response with SNX-11, an N-type channel blocker, in a rodent model of focal cerebral ischaemia using diffusion-weighted MRI. Brain Res. 739, 36–45.

Yu, A.C., Chan, P.H., Fishman, R.A., 1987. Arachidonic acid inhibits uptake of Glu and glutamine but not GABA in cultured cerebellar granule cells. J. Neurosci. Res. 17, 424–427.

Zhao, Q., Smith, M.L., Siesjo, B.K., 1994. The omega-conopeptide SNX-111, an N-type calcium-channel blocker, dramatically ameliorates brain damage due to transient focal ischaemia. Acta Physiol. Scand. 150, 459–461.