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ω-Agatoxin IVA-sensitive Ca^{2+} channel blocker, α-eudesmol, protects against brain injury after focal ischemia in rats

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Abstract

ω-Agatoxin IVA-sensitive Ca^{2+} channels have been thought to be involved in physiological excitatory amino acid glutamate release and these channels may also contribute to the development of ischemic brain injury. Recently, we demonstrated that α-eudesmol from *Juniperus virginiana* Linn. (Cupressaceae) inhibits potently the presynaptic ω-agatoxin IVA-sensitive Ca^{2+} channels. In the present study, we investigated the effects of α-eudesmol on brain edema formation and infarct size determined after 24 h of reperfusion following 1 h of middle cerebral artery occlusion in rats. We first found that α-eudesmol concentration-dependently inhibited glutamate release from rat brain synaptosomes and that its inhibitory effect was Ca^{2+}-dependent. In the middle cerebral artery occlusion study, intracerebroventricular (i.c.v.) treatment with α-eudesmol significantly attenuated the post-ischemic increase in brain water content. α-Eudesmol also significantly reduced the size of the infarct area determined by triphenyltetrazolium chloride staining after 24 h of reperfusion. Using a microdialysis technique, we further demonstrated that α-eudesmol inhibits the elevation of the extracellular concentration of glutamate during ischemia. From these results, we suggest that α-eudesmol displays an ability to inhibit exocytotic glutamate release and to attenuate post-ischemic brain injury. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: ω-Agatoxin IVA-sensitive Ca^{2+} channel; α-Eudesmol; Glutamate release; Synaptosome; Microdialysis; Focal ischemia

1. Introduction

In physiological transmitter release, Ca^{2+} influx through voltage-dependent Ca^{2+} channels is well known to be the trigger for exocytotic release from nerve terminals (Mulkey and Zucker, 1991). ω-Agatoxin IVA, a peptide toxin isolated from the funnel web spider *Agelenopsis aperta*, has been widely reported to markedly inhibit synaptosomal Ca^{2+} uptake, synaptosomal transmitter release and synaptic transmission (Mintz et al., 1992a,b; Turner et al., 1992; Uchitel et al., 1992; Luebke et al., 1993; Iraq et al., 1994; Momiyama and Takahashi, 1994; Regehr and Mintz, 1994; Wheeler et al., 1994; Turner and Dunlap, 1995). Although high-threshold Ca^{2+} channels, L-type (dihydropyridine-sensitive), N-type (ω-conotoxin GVIA-sensitive) and P/Q-types (ω-agatoxin IVA-sensitive), have been mainly identified in neuronal cells (Llinas et al., 1992; Usowicz et al., 1992; Zhang et al., 1993; Thomas et al., 1994; Dunlap et al., 1995; Graham and Burgoyne, 1995; Maubecin et al., 1995; Randall and Tsien, 1995), physiological transmitter release is suggested to be closely coupled to the ω-agatoxin IVA-sensitive P/Q-type Ca^{2+} channel.

In cerebral ischemia, massive amounts of excitatory transmitter glutamate are released from cells into the extracellular space (Benveniste et al., 1984; Hillered et al., 1989; Butcher et al., 1990; Katayama et al., 1991), and the glutamate exacerbates neuronal damage by activating *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors (Simon et al., 1984; Choi, 1988). Pathological glutamate release under ischemic conditions is thought to result from not only Ca^{2+}-dependent exocytotic release from nerve terminals but also Ca^{2+}-independent release, which has been suggested to be mediated by the reversed glutamate transporter from nerve terminals or glial cells (Attwell et al., 1993; Gemba et al., 1994). On the other hand, pathological Ca^{2+}-dependent glutamate release has been previously suggested to be mediated by the N-type Ca^{2+} chan-

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nel in studies using ω-conotoxins, which protect against post-ischemic brain injury (Buchan et al., 1994; Yamada et al., 1994; Zhao et al., 1994; Syunya et al., 1995; Yenari et al., 1996). We recently demonstrated that ω-agatoxin IVA protects against brain injury after focal ischemia in rats, which suggested that the ω-agatoxin IVA-sensitive Ca^{2+} channel may also be coupled to pathological excitatory amino acid release and be involved in the development of the brain injury (Asakura et al., 1997).

Recently, we demonstrated that α-eudesmol from *Juniperus virginiana* Linn. (Cupressaceae) blocks presynaptic ω-agatoxin IVA-sensitive Ca^{2+} channels in rat brain synaptosomes with an IC_{50} value of 2.6 μM (Asakura et al., 1999). The inset of Fig. 1 shows the chemical structure of α-eudesmol. Electrophysiologically, α-eudesmol has also been demonstrated to inhibit ω-agatoxin IVA-sensitive (P-type) Ca^{2+} channels with an IC_{50} value of 3.6 μM, ω-conotoxin GVIA-sensitive (N-type) Ca^{2+} channels with an IC_{50} value of 6.6 μM, and dihydropyridine-sensitive (L-type) Ca^{2+} channels with an IC_{50} value of about 45 μM. Although α-eudesmol is not specific for ω-agatoxin IVA-sensitive Ca^{2+} channels at higher concentrations, the inhibitory effect on the ω-agatoxin IVA-sensitive Ca^{2+} channel was more potent than that on the other Ca^{2+} channels. The funnel web spider toxin (polyamine) from *A. aperta*, FTX, is the first non-peptide to block ω-agatoxin IVA-sensitive Ca^{2+} channels (Uchitel et al., 1992). However, the inhibitory effects on the P-type, the N-type, and the L-type Ca^{2+} channels are poorly selective and very weak (Norris et al., 1996). Although fluspirilene has also been reported to potently inhibit the P-type Ca^{2+} channel with an IC_{50} value of 6 μM, the inhibitory effect on the L-type Ca^{2+} channel was most potent among the above three Ca^{2+} channels (Sah and Bean, 1993; Grantham et al., 1994). We consider that α-eudesmol is a non-peptide compound, which blocks potently and selectively the ω-agatoxin IVA-sensitive Ca^{2+} channel in comparison with the low-molecular weight compounds already reported as ω-agatoxin-IVA-sensitive Ca^{2+} channel blockers.

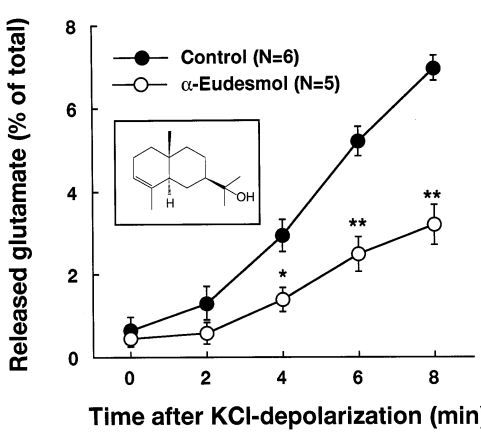

Fig. 1. Inhibitory effects of α-eudesmol on KCl-evoked glutamate release from rat brain synaptosomes. Depolarization was evoked with 60 mM KCl for 8 min. α-Eudesmol (45 μM) was added 6 min before the start of KCl depolarization. Cumulative glutamate release was determined over time as the sum of the release in all fractions up to the indicated point and was plotted for α-eudesmol and vehicle. Data are presented as means ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle (unpaired *t*-test). The inset shows the chemical structure of α-eudesmol.

In the present study, to further address the pathological role of the ω-agatoxin IVA-sensitive Ca^{2+} channel, we evaluated the effect of α-eudesmol against the development of post-ischemic brain injury. We first demonstrate that α-eudesmol attenuates cerebral edema formation and reduces cerebral infarct size determined after 24 h of reperfusion following 1 h of middle cerebral artery occlusion in rats. We also demonstrate, using a microdialysis technique, that α-eudesmol inhibits not only the release of glutamate from nerve terminals using rat brain synaptosomes but also the elevation of extracellular glutamate concentration during cerebral ischemia.

2. Materials and methods

2.1. Animals

We used Sprague–Dawley rats (Crj, Japan) in in vitro experiments using synaptosomes and Wistar rats (Slc, Japan) in in vivo experiments. They were housed in a room with an ambient temperature of 24 ± 1°C and humidity of 55 ± 5% under a 12-h light/dark cycle. They were allowed free access to food and water and were cared for in compliance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

2.2. Preparation of synaptosomes

Rats (21–28 days old) were anesthetized with ether and decapitated. The synaptosomes were prepared from whole brains (except for the cerebellum) in accordance with previous reports (Kanemasa et al., 1995; Asakura et al., 1999). Whole brains were homogenized with isolation medium (320 mM sucrose, 15 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 5 mM ethylene diamine tetraacetic acid (EDTA), pH 7.4). The homogenate was first centrifuged for 10 min at 3,000 × *g*. The pellet was discarded and the supernatant was recentrifuged for 20 min at 12,500 × *g*. The pellet was resuspended in fresh isolation medium, layered onto a discontinuous gradient of 3%, 10%, 15% and 23% Percoll (Sigma) in the isolation medium, and centrifuged at 32,500 × *g* for 5 min. Synaptosomes layered on 23% Percoll were stored at 1 mg/ml in basal saline (145 mM NaCl, 3.1 mM KCl, 1.2 mM $MgCl_2$, 0.4 mM KH_2PO_4, 0.5 mM $CaCl_2$, 10 mM glucose, 10 mM Tris [hydroxymethyl]-aminomethane hydrochloride (Tris), pH 7.4). All of the above procedures were performed at 4°C.

2.3. Measurement of glutamate release from synaptosomes

Synaptosomes were layered on cellulose nitrate filters mounted in a superfusion system and then superfused with basal saline at a flow rate of 0.6 ml/min. After a 40-min equilibration period, synaptosomes were pre-incubated in saline for 6 min. Synaptosomes were further depolarized for 8 min with 60 mM KCl. The first 6-min fraction was collected as the basal release and then four 2-min fractions were collected as the K^+-evoked release into ice-cold plastic tubes containing perchloric acid. Synaptosomes were treated with α-eudesmol throughout the pre-incubation and depolarizing periods. In Ca^{2+}-free experiments, synaptosomes were first pre-incubated in the basal Ca^{2+}-free medium (without $CaCl_2$) containing 1 mM ethylene glycol bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) for 6 min and then stimulated with 60 mM KCl (Ca^{2+}-free medium) for 8 min as mentioned above. After the sampling procedure, the amount of glutamate in the perfusate was measured by high-performance liquid chromatography (HPLC) with an electrochemical detection method using *o*-phthalaldehyde (Nacalai Tesque) and a Cosmosil packed column (5C 18, 150 × 4.6 mm; Nacalai Tesque). The glutamate released into the perfusate is expressed as the percentage of total synaptosomal glutamate content measured after osmotic rupture and sonication.

2.4. Middle cerebral artery occlusion

A transient focal cerebral ischemia rat model was prepared by middle cerebral artery occlusion as described in previous reports (Matsuo et al., 1994; Asakura et al., 1997). Adult male Wistar rats (270–320 g), specific-pathogen-free, were anesthetized with a gas mixture of 70% N_2O, 30% O_2, and 2% halothane. After a median incision of the neck skin, the right external carotid artery was dissected. An 18-mm-long 4-0 nylon thread (Nitcho Kogyo) was precoated with silicone (Xantpren, Bayer Dental) mixed with a hardener (Optosil Activator, Bayer Dental) to increase the thickness of the distal half. The coated thread was inserted from the lumen of the right external carotid artery to the right internal carotid artery to occlude the origin of the right middle cerebral artery. Body temperature was maintained at 37°C with a heating pad. After surgery, the anesthesia was discontinued and the rats were allowed free access to food and water until the next procedure. Neurological deficits characterized by severe left-sided hemiparesis and right Horner's syndrome were used as criteria for ischemic insult. Ischemic animals exhibited severe hemiparesis with counterclockwise circling and rolling to the left side. After 1 h of middle cerebral artery occlusion, the thread was removed to allow reperfusion of the ischemic area via the right common carotid artery. Sham-operated rats were subjected to the same procedure expect for the middle cerebral artery occlusion.

2.5. Measurement of brain water content

Brain water content was measured in accordance with previous reports (Matsuo et al., 1994; Asakura et al., 1997). Adult male Wistar rats were decapitated after 24 h of reperfusion following 1 h of transient middle cerebral artery occlusion. Samples of ischemic hemisphere were taken from the cerebral cortex perfused by the anterior cerebral artery (the anterior cerebral artery area), the cerebral cortex perfused by the middle cerebral artery (the middle cerebral artery area) and the caudate putamen. The ischemic area was clarified by transcardiac perfusion of the ischemic rats with carbon black as reported previously (Longa et al., 1989; Matsuo et al., 1994; Asakura et al., 1997). The colorless area was considered to correspond to the area supplied by the occluded middle cerebral artery. The cerebral cortex corresponding to the colorless area was taken as the middle cerebral artery area, and the rest of the cortex was taken as the anterior cerebral artery area. Fig. 3A shows the sampling areas (the middle cerebral artery area, the anterior cerebral artery area, caudate putamen). The water content of brain samples at 24 h after reperfusion was calculated by the dry-weight method: 100 × (wet weight − dry weight)/wet weight (%).

2.6. Measurement of the size of the infarct area

To measure the size of the infarct area, adult male Wistar rats were perfused with physiological saline containing 0.2% heparin after 24 h of reperfusion (Matsuo et al., 1994; Asakura et al., 1997). The brains were removed from the skulls, cut into 1-mm coronal sections and immersed in 2% triphenyltetrazolium chloride (TTC) solution at 37°C for 30 min. The colorless areas with TTC staining, which reflect mitochondrial damage, were quantified as infarct areas by an image analyzer system (Kontron M14, Zeiss).

2.7. Microdialysis experiments

Adult male Wistar rats were anesthetized with a gas mixture of 70% N_2O, 30% O_2, and 2% halothane. Through a burr hole (coordinates: 0.8 mm anterior to the bregma, 5 mm lateral to the right side of the bregma), a cannula-type dialysis probe (molecular cut-off 50,000; outer diameter of 200 μm; membrane length of 2 mm) was stereotactically inserted to a depth of 3 mm from the skull surface. The probe was fixed to the skull with dental cement and screws. After implantation of the dialysis probe, anesthesia was discontinued. The rats were allowed free access to food and water until the next procedure. These rats were subjected to dialysis experiments 24 h after implantation. All experiments were carried out with the animals under freely moving conditions except during middle cerebral artery occlusion surgery. The dialysis probe was connected

Table 1
Concentration-dependent inhibition by α-eudesmol of KCl-evoked glutamate release from rat brain synaptosomes
Depolarization was evoked with 60 mM KCl for 6 min. α-Eudesmol (0.45–45 μM) was added 6 min before the start of the depolarization. Data are presented as means ± S.E.M. from four to seven experiments

Treatment	Released glutamate (% of total)
Vehicle	6.24 ± 0.29
0.45 μM α-Eudesmol	5.05 ± 0.26
4.5 μM α-Eudesmol	4.59 ± 0.27[a]
45 μM α-Eudesmol	3.25 ± 0.19[a]

[a] $P < 0.01$ vs. vehicle (Dunnett's multiple range test).

to a microinfusion pump (CMA/100, Carnegie Medicin, Sweden) via a fused silica tube with an outer diameter of 75 μm and continuously perfused with artificial cerebrospinal fluid (aCSF) at 2 μl/min. After a 90-min equilibration period, the middle cerebral artery was occluded. α-Eudesmol (45 μM) was continuously infused into the aCSF for 100 min (during the last 20-min equilibration, 60-min ischemic and 20-min reperfusion periods). Samples were collected in an ice-cold plastic tube containing perchloric acid every 20 min throughout the present study (fractions before and after drug application, fractions after ischemia and reperfusion). At the end of the experiment, Evans blue (2%) was administered via the dialysis catheter to confirm the probe placement. The amount of glutamate released into the perfusate was measured by HPLC (see the measurement of glutamate release from synaptosomes). Methionine sulphone (Wu et al., 1995) was added as an internal standard to each sample in this study. All data are expressed as percentages of the basal value before drug application (percentage of basal).

2.8. Drug application

α-Eudesmol, isolated from *J. virginiana* Linn. (Shionogi, Japan), was dissolved in phosphate buffer containing 0.1% dimethyl sulfoxide, which was used as the vehicle. For intracerebroventricular (i.c.v.) injection, one burr hole was drilled through the cranium, 1.6 mm lateral to the right side of the bregma. Vehicle or α-eudesmol (volume 2 μl) was i.c.v. injected via a stainless-steel needle with a microsyringe inserted stereotactically into the right lateral ventricle. In the middle cerebral artery occlusion experiments (brain edema and infarct size), α-eudesmol or vehicle was injected 10 min before middle cerebral artery occlusion.

2.9. Statistics

All data are presented as means ± standard error (S.E.M.). For statistical analysis, Dunnett's multiple range test, Tukey's test or unpaired *t*-test was used. Differences were considered significantly at a value of $P < 0.05$.

3. Results

3.1. Inhibitory effects of α-eudesmol on K^+-evoked glutamate release from synaptosomes

To clarify whether α-eudesmol inhibits excitatory transmitter glutamate release from nerve terminals, we evaluated the effect of α-eudesmol on endogenous glutamate release from rat brain synaptosomes using a superfusion system. The inset in Fig. 1 shows the chemical structure of α-eudesmol. It has been reported that α-eudesmol at 45 μM markedly blocks K^+-evoked Ca^{2+} uptake into the synaptosomes (Asakura et al., 1999). In the present study, we first demonstrated that 45 μM α-eudesmol inhibited the K^+-evoked glutamate release (Fig. 1), however, it did not affect the basal glutamate release measured prior to the K^+-evoked depolarization. Table 1 shows the concentration-dependent inhibition by α-eudesmol of glutamate release. The glutamate release from the synaptosomes is reported to be mediated by Ca^{2+}-dependent and independent pathways (Turner et al., 1992; Attwell et al., 1993). The inhibitory effect of α-eudesmol under Ca^{2+}-free conditions was further evaluated to address its Ca^{2+}-dependence (Fig. 2). The Ca^{2+}-free condition itself significantly attenuated the K^+-evoked glutamate release and its extent was similar to that measured in the presence of α-eudesmol. α-Eudesmol did not cause any additive inhibitory effect under Ca^{2+}-free conditions. These results indicate that α-eudesmol inhibited Ca^{2+}-dependent glutamate release from nerve terminals.

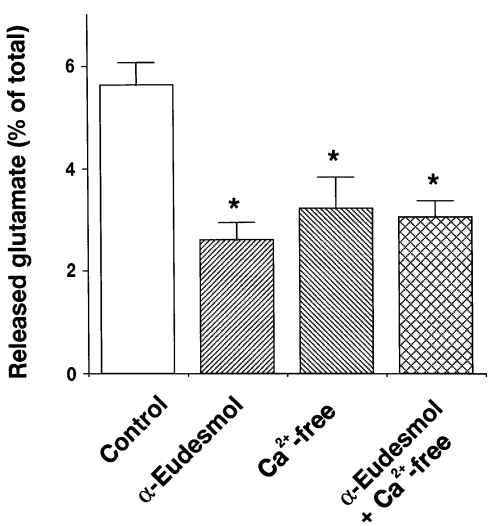

Fig. 2. Ca^{2+}-dependence of α-eudesmol-induced inhibition of KCl-evoked glutamate release from rat brain synaptosomes. Depolarization was evoked with 60 mM KCl for 8 min. α-Eudesmol (45 μM) was added 6 min before the start of KCl depolarization. In Ca^{2+}-free experiments, synaptosomes were superfused with the Ca^{2+}-free medium (without $CaCl_2$) containing 1 mM EGTA. Data are presented as means ± S.E.M. from five to six experiments. * $P < 0.01$ vs. vehicle (Tukey's test).

3.2. Protective effects of α-eudesmol on post-ischemic brain edema

To evaluate the effects of α-eudesmol on post-ischemic brain edema formation in rats, we measured the brain water content after 24 h of reperfusion following 1 h of middle cerebral artery occlusion. Brain samples of ischemic hemisphere were taken from the cerebral cortex perfused by the anterior cerebral artery (the anterior cerebral artery area), the cerebral cortex perfused by the middle cerebral artery (the middle cerebral artery area) and the caudate putamen (Fig. 3A). The water content in the vehicle-control group significantly increased from 79.48 ± 0.19% (sham operation) to 82.43 ± 0.30% (vehicle, $P <$ 0.01) in the anterior cerebral artery area, from 79.48 ± 0.14% (sham operation) to 85.58 ± 0.35% (vehicle, $P <$ 0.01) in the middle cerebral artery area and from 75.92 ± 0.22% (sham operation) to 84.09 ± 0.35% (vehicle, $P <$ 0.01) in the caudate putamen after 24 h of reperfusion (Fig. 3B). Treatment with α-eudesmol at 30 pmol i.c.v. clearly and significantly attenuated the increase in water content in all the above sampling areas. Treatment with 3 pmol of α-eudesmol significantly attenuated the post-ischemic increase in water content only in the anterior cerebral artery area. Treatment with 300 pmol and 3 nmol of α-eudesmol significantly attenuated them only in the middle cerebral artery area and caudate putamen. Thus, α-eudesmol inhibited the brain edema formation dose-dependently up to 30 pmol but its inhibitory effect was saturated or weak at doses higher than 300 pmol compared its effect at 30 pmol.

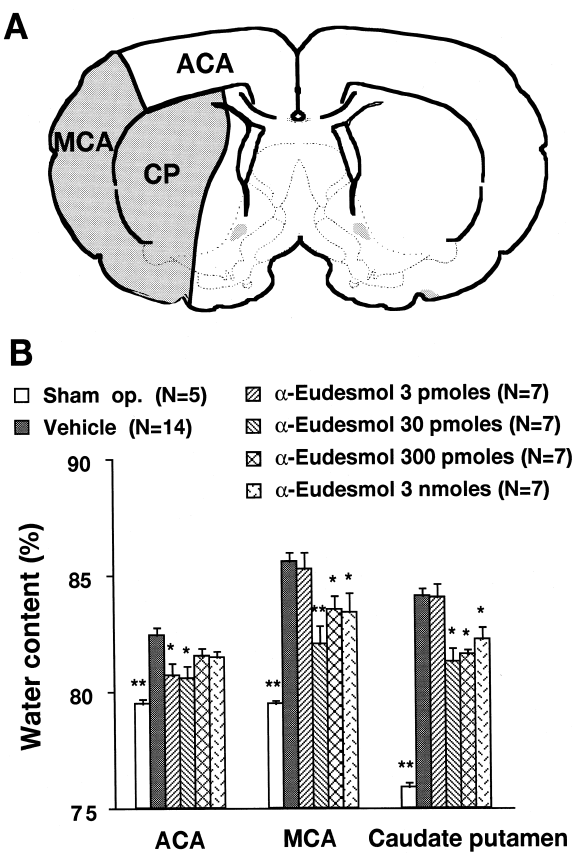

Fig. 3. (A) Diagram of anatomic regions in a coronal section through the caudate putamen of the rat. Tissue was taken from these regions for the measurement of brain water content. ACA, frontoparietal cortex supplied by the anterior cerebral artery; MCA, frontoparietal cortex supplied by the middle cerebral artery; CP, caudate putamen. The shaded area represents a typical ischemic area. (B) Protective effects of α-eudesmol on brain water content after 24-h reperfusion following 1 h of middle cerebral artery occlusion. α-Eudesmol was injected i.c.v. 10 min before ischemia. Water content was measured by the dry-weight method for tissue samples from the ACA, the MCA and the CP in α-eudesmol or vehicle-treated rats and sham-operated rats. Data are presented as means ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle (Dunnett's multiple range test).

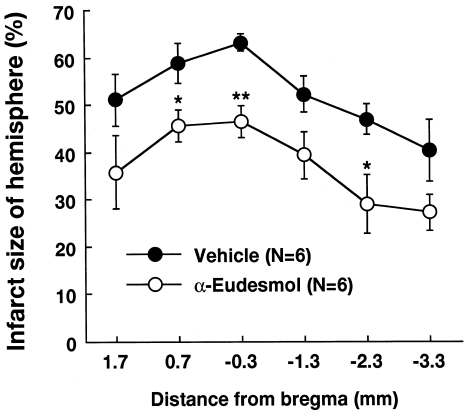

Fig. 4. Protective effects of α-eudesmol on infarct size (expressed as a percentage of the occluded hemisphere) of cerebrum coronal level 1.7 mm anterior (bregma 1.7), 0.7 mm anterior (bregma 0.7), 0.3 mm posterior (bregma −0.3), 1.3 mm posterior (bregma −1.3), 2.3 mm posterior (bregma −2.3), 3.3 mm posterior (bregma −3.3) at 24 h after reperfusion following 1-h middle cerebral artery occlusion. α-Eudesmol (30 pmol) was injected i.c.v. 10 min before ischemia. Data are presented as means ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle (unpaired *t*-test).

3.3. Protective effects of α-eudesmol on post-ischemic brain infarction

We investigated the effects of α-eudesmol on the infarct size measured after 24 h of reperfusion following 1 h of middle cerebral artery occlusion (Fig. 4). The ischemic stress markedly caused brain infarction in vehicle-treated rats, which was consistent with previous reports (Matsuo et al., 1994; Asakura et al., 1997). α-Eudesmol at 30 pmol i.c.v., which significantly attenuated post-ischemic brain edema as shown in Fig. 3, significantly reduced the infarct size as shown in Fig. 4 (bregma 0.7 mm: vehicle, 58.67 ± 4.19%; α-eudesmol, 45.61 ± 3.31%, $P < 0.05$; bregma −0.3 mm: vehicle, 63.02 ± 1.87%; α-eudesmol, 46.38 ± 3.26%, $P < 0.01$; bregma −2.3 mm: vehicle, 46.80 ± 3.25%; α-eudesmol, 28.98 ± 6.27%, $P < 0.05$). In the other areas in the cerebrum, α-eudesmol tended to reduce the infarct size.

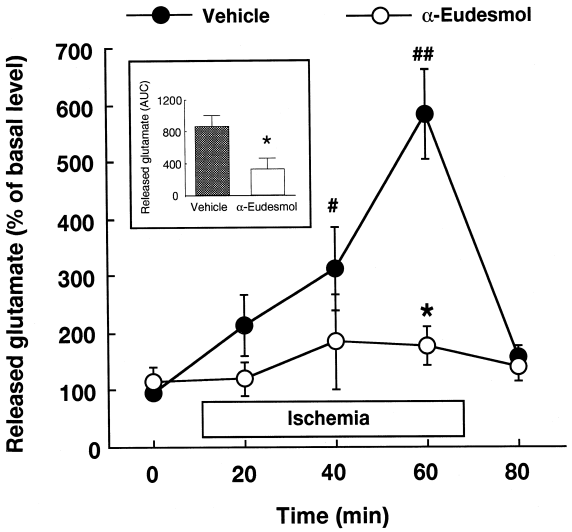

Fig. 5. Inhibitory effects of α-eudesmol on the elevation of glutamate level in the dialysis perfusate of rats with middle cerebral artery occlusion. α-Eudesmol was continuously infused in the dialysis perfusate from 20 min prior to 1 h of middle cerebral artery occlusion. Data were calculated by using basal measurements taken before drug application (percentage of basal) and presented as means ± S.E.M. ($N = 6$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. basal (Dunnett's multiple range test). $^{*}P < 0.05$, vs. vehicle (unpaired *t*-test). The inset shows the inhibitory effect of α-eudesmol on the total glutamate release during ischemic periods ($^{*}P < 0.05$, unpaired *t*-test).

3.4. Inhibitory effects of α-eudesmol on glutamate release during focal cerebral ischemia

To clarify whether α-eudesmol inhibits the excess glutamate release during cerebral ischemia, we monitored the extracellular glutamate level using an in vivo microdialysis technique (Fig. 5). The extracellular glutamate level in the vehicle-control group was significantly elevated during middle cerebral artery occlusion in comparison to the basal level, and then returned to the basal level after reperfusion. Fig. 5 shows that α-eudesmol significantly attenuated the elevation of the glutamate level during the indicated ischemic period ($P < 0.05$). Furthermore, as shown in the inset in Fig. 5, α-eudesmol also significantly reduced the total ischemic glutamate release compared with the effect of vehicle treatment. α-Eudesmol did not produce a significant change in the basal glutamate level prior to cerebral ischemia.

4. Discussion

The P/Q-type Ca^{2+} channel blocker ω-agatoxin IVA has been demonstrated to potently inhibit exocytotic glutamate release from nerve terminals under physiological conditions in mammals (Mintz et al., 1992a,b; Turner et al., 1992; Uchitel et al., 1992; Luebke et al., 1993; Iraq et al., 1994; Momiyama and Takahashi, 1994; Regehr and Mintz, 1994; Wheeler et al., 1994; Turner and Dunlap, 1995). Massive amounts of the excitatory amino acid glutamate are thought to be released in the ischemic period, and ischemic brain injury becomes more serious due to the action of such amino acids (Benveniste et al., 1984; Simon et al., 1984; Choi, 1988; Hillered et al., 1989; Katayama et al., 1991; Buchan et al., 1994). Previously, Small et al. (1995) reported the post-ischemic protective effect of ω-agatoxin IVA in hippocampal slices using an in vitro ischemia experiment. Furthermore, ω-conotoxin MVIIC, which blocks not only N-type but also P/Q-type Ca^{2+} channels, has been also reported to inhibit pathological excitatory amino acid release during ischemia (Wu et al., 1995). Based on the above suggestions, we hypothesized that blockade of the ω-agatoxin IVA-sensitive Ca^{2+} channel may inhibit the excess release of excitatory neurotransmitter during ischemia in vivo and exhibit a post-ischemic protective effect. We have previously demonstrated that ω-agatoxin IVA indeed offers protection against post-ischemic brain injury in middle cerebral artery-occluded rats (Asakura et al., 1997).

In the present study, we investigated the effects of α-eudesmol, which potently blocks presynaptic ω-agatoxin IVA-sensitive Ca^{2+} channels, on glutamate release from nerve terminals and on post-ischemic brain injury. We showed that α-eudesmol inhibits Ca^{2+}-dependent glutamate release from synaptosomes, attenuates brain edema formation and reduces the infarct size in rats with middle cerebral artery occlusion. Furthermore, we also demonstrated that α-eudesmol significantly inhibits the elevation of extracellular glutamate concentration during middle cerebral artery occlusion using the microdialysis technique.

In the present study, the inhibitory effects of α-eudesmol on brain edema formation were dose-dependent up to 30 pmol but saturated or weaker at doses higher than 300 pmol. Thus, α-eudesmol exhibited bell-shaped inhibitory effects on post-ischemic edema formation. The ω-agatoxin IVA-sensitive Ca^{2+} channel is thought to be coupled to the release of not only excitatory amino acid transmitters but also inhibitory amino acid transmitters under physiological conditions (Iraq et al., 1994; Momiyama and Takahashi, 1994). Massive amounts of inhibitory neurotransmitter release during cerebral ischemia may play a protective role in pathological conditions, unlike excitatory amino acid release (Ravindran et al., 1994). Taking these suggestions into consideration, a decrease in inhibitory transmitter release caused by blockade of the ω-agatoxin IVA-sensitive Ca^{2+} channel during ischemia may attenuate the protective effects of α-eudesmol at its higher dose. Previously, we reported a similar bell-shaped response with ω-agatoxin IVA, which inhibits edema formation in middle cerebral artery-occluded rats (Asakura et al., 1997). Our present results agree with the previous results for ω-agatoxin IVA. This is reasonable because α-eudesmol has a similar profile to ω-agatoxin IVA with respect to the ability to inhibit the same Ca^{2+} channel.

In the present study, we found no effect of α-eudesmol on neurological behavior (data not shown). ω-Agatoxin

IVA, at a higher dose (over 100 pmol), caused convulsions (Asakura et al., 1997). Since the inhibitory effect of ω-agatoxin IVA (IC_{50} value, 80 nM), assessed in synaptosomal $^{45}Ca^{2+}$ uptake experiments, is more potent than that of α-eudesmol (IC_{50} value, 2.6 μM) (Asakura et al., 1999), it is reasonable that ω-agatoxin IVA more potently affects neurological behavior under physiological conditions than α-eudesmol does. However, the post-ischemic protective effect of ω-agatoxin IVA was less potent than its behavioral effect, since ω-agatoxin IVA had protective effects at 10 to 100 pmol on brain edema formation (Asakura et al., 1997), which is almost equipotent with α-eudesmol. The ω-agatoxin IVA-induced channel blockade is reported to be reversed by strong depolarization (Mintz et al., 1992a,b). As the membrane potential under ischemic conditions is thought to be more depolarized than under physiological conditions, ω-agatoxin IVA may be less effective under pathological conditions than under physiological conditions. It has also been reported that low-molecular weight compounds such as U50488 and fluspirilene block the ω-agatoxin IVA-sensitive P-type Ca^{2+} channel in a voltage-dependent and frequency-dependent manner (Sah and Bean, 1993; Kanemasa et al., 1995). These reports show that the channel-blocking effects of these compounds are more potent under pathological conditions than under physiological conditions. Although the blocking profile of α-eudesmol remains unknown, it is very likely to possess the above-mentioned characteristics on the blockade of the ω-agatoxin IVA-sensitive P-type Ca^{2+} channel.

Lingenhöhl et al. (1997) reported that daurisoline, which blocks the P-type Ca^{2+} channel, did not attenuate the brain edema induced by middle cerebral artery occlusion. They concluded that the P-type Ca^{2+} channel is not involved in post-ischemic brain injury. However, we consider that the absence of the post-ischemic protective effect may have been due to insufficient attenuation of the excess glutamate release during ischemia, since the blocking activity of daurisoline (Lu et al., 1994) on the P-type Ca^{2+} channel is very weak, with an IC_{50} value of 35.6 μM, compared with that of ω-agatoxin IVA and α-eudesmol. Since the Q-type Ca^{2+} channel is suggested to be involved in physiological synaptic transmission as well as in pathological excitatory amino acid release (Wheeler et al., 1994; Lingenhöhl et al., 1997), the blocking activity of daurisoline may affect the potency of its post-ischemic protective effect. Indeed, ω-agatoxin IVA is reported to inhibit both P-type and Q-type Ca^{2+} channels (Zhang et al., 1993). We previously showed electrophysiologically that α-eudesmol inhibits the P-type Ca^{2+} channel (Asakura et al., 1999), but its inhibitory effect on the Q-type Ca^{2+} channel is not known in detail. Although it is difficult to clarify which of these Ca^{2+} channels is important in the development of brain injury, we believe that α-eudesmol as well as ω-agatoxin IVA and daurisoline should be useful for clarifying it.

Brain hypothermia is known to attenuate post-ischemic brain injury (Busto et al., 1987; Chen et al., 1992; Morikawa et al., 1992). We also studied the effect of α-eudesmol on brain temperature in conscious rats to exclude the possibility that the α-eudesmol-induced post-ischemic neuroprotective effect is produced by hypothermia. We found that α-eudesmol, at a dose which exhibited protection against the post-ischemic brain injury, did not produce hypothermia (data not shown).

In conclusion, we demonstrated that α-eudesmol protects against the development of post-ischemic brain injury. Furthermore, synaptosomal and microdialysis experiments showed that α-eudesmol has the ability to inhibit exocytotic glutamate release. This suggests that its inhibition of presynaptic glutamate release during ischemia may produce a neuroprotective effect. However, further study is necessary to confirm its therapeutic efficacy against post-ischemic brain injury. Since ω-agatoxin IVA protected against post-ischemic brain injury and α-eudesmol potently inhibits presynaptic ω-agatoxin IVA-sensitive Ca^{2+} channels rather than other Ca^{2+} channels (Asakura et al., 1997, 1999), we suggest that the neuroprotection by α-eudesmol in the present study is produced by ω-agatoxin IVA-sensitive Ca^{2+} channel blockade. However, taking the specificity of α-eudesmol at high concentrations into consideration, we cannot exclude the possibility that the blockade of other Ca^{2+} channels may be also involved in its neuroprotection mechanism. In addition to the development of post-ischemic brain injury, as ω-agatoxin IVA-sensitive Ca^{2+} channels seem to be important for the nociceptive process by signals from inflamed tissue (Norris et al., 1996; Diaz and Dickenson, 1997; Nebe et al., 1997), α-eudesmol may be also effective against the development of pain and neurogenic inflammation.

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