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DY-9760e, a novel calmodulin antagonist, reduces brain damage induced by transient focal cerebral ischemia

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Abstract

Perturbations in Ca^{2+} homeostasis have been proposed to lead to neuronal damage after cerebral ischemia. DY-9760e (3-[2-[4-(3-chloro-2-methylphenyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1*H*-indazole dihydrochloride 3.5 hydrate) is a novel calmodulin antagonist. In this study, we examined the effects of DY-9760e on brain damage in rats subjected to transient (1 h) focal cerebral ischemia. DY-9760e (0.25–1.00 mg kg^{-1} h^{-1}) was intravenously infused for 6 h, starting 1 h after middle cerebral artery occlusion. Treatment with DY-9760e 0.25, 0.50 and 1.00 mg kg^{-1} h^{-1} reduced infarct volume by 30, 42 ($P < 0.05$), and 60% ($P < 0.05$), respectively. Furthermore, the effect of DY-9760e on ischemia-induced fodrin breakdown was examined in the same model. Pronounced fodrin breakdown was observed in the cerebral cortex and striatum at 24 h after ischemia. DY-9760e caused potent suppression of fodrin breakdown in the cerebral cortex at the same doses as those that had a protective action against cerebral infarction. From these results DY-9760e may have a therapeutic effect against cerebral ischemic damage in the acute stage.

Keywords: DY-9760e; Calmodulin antagonist; Ischemia, transient, focal; Cerebral infarction; Fodrin

1. Introduction

An excessive elevation of intracellular Ca^{2+} concentration is known to play an important role in the pathological events following cerebral ischemia (Choi, 1988, 1995; Meldrum and Garthwaite, 1990; Mitani et al., 1993). Elevation of the Ca^{2+} concentration in neurons disturbs the ionic balance and activates various Ca^{2+}-dependent enzymes and Ca^{2+}-binding proteins.

Calmodulin is a major Ca^{2+}-binding protein in the central nervous system (Zhou et al., 1985; James et al., 1995). Calmodulin is implicated in a variety of cell functions by activation of calmodulin-dependent enzymes, such as phosphodiesterase, protein kinases, protein phosphatase, and nitric oxide (NO) synthase (James et al., 1995). Ca^{2+} overload in neurons induced by ischemic insult may overactivate Ca^{2+}/calmodulin-dependent pathways, and these events may lead to irreversible neuronal damage. In fact, it has been reported that persistent Ca^{2+}/calmodulin-binding in neurons is associated with neuronal damage in an ischemic model in rats (Picone et al., 1989), and that calmodulin antagonists, calmidazolium (1-[bis-(4-chlorophenyl)methyl]-3-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)methoxy]-ethyl]-1*H*-imidazolium chloride) and W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide), protect neuronal cells against hypoxia-induced neuronal injury in organotypic cultures (Sun et al., 1997). These studies suggest that calmodulin plays a critical role in mediating some of the biochemical events leading to neuronal damage.

Among the calmodulin-binding proteins, fodrin, a major cytoskeletal protein lying under the plasma membrane in the brain, is degraded by a Ca^{2+}-dependent protease, calpain (Siman et al., 1984), after cerebral ischemia (Saido et al., 1993; Roberts-Lewis et al., 1994; Bartus et al., 1995). Furthermore, it has been shown that calmodulin stimulates the degradation of fodrin by calpain (Seubert et al., 1987; Johnson et al., 1991).

We previously reported the pharmacological characterization and cytoprotective effect of DY-9760e (3-[2-[4-(3-

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chloro-2-methylphenyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1*H*-indazole dihydrochloride 3.5 hydrate), a novel calmodulin antagonist (Sugimura et al., 1997). We show here that DY-9760e can reduce infarct volume and the breakdown of fodrin in transiently middle cerebral artery-occluded rats.

2. Materials and methods

2.1. Animals

We used male Wistar rats (Clea Japan, Tokyo, Japan) weighing 240–280 g at surgery. Animals were housed in cages and given food (F-2; Funabashi Farm, Chiba, Japan) and tap water ad libitum, and were kept in a regulated environment (23 ± 2°C, 55 ± 5% relative humidity) under a 12-h light/dark cycle (on 8:00 to 20:00). All experimental procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Daiichi Pharmaceutical.

2.2. Transient focal ischemia model

Animals were anesthetized by inhalation of 2% halothane in 70% nitrous oxide and 30% oxygen. Surgery was carried out under a heating lamp to maintain body temperature. Transient focal cerebral ischemia was induced as described by Koizumi et al. (1986) with minor modifications. In brief, a surgical midline incision was made to expose the left common, internal and external carotid arteries. The external carotid and occipital arteries were ligated. The common carotid artery was closed by a ligature, and a suture was tied loosely around the internal carotid artery. A small incision was then made in the common carotid artery, and a 22-mm length of 3-0 monofilament nylon suture (Matuda Ika Kogyo, Tokyo, Japan) coated with poly-L-lysine (Belayev et al., 1995) was inserted into the internal carotid artery. The occluder filament was advanced to close to the origin of the middle cerebral artery. The anesthesia was then discontinued, and the animals were returned to their cages after the surgery. The animals were anesthetized for less than 8 min, because in a preliminary study we showed that physiological parameters (blood pressure, arterial blood gases and rectal temperature) changed little in this period (data not shown). The rats remained awake during the ischemic period, and their behavior was checked for consciousness and neurological deficits (forelimb flexion). Animals with sustained disturbance of consciousness, with intracerebral bleeding, and without forelimb flexion were excluded from further study. After 1 h of middle cerebral artery occlusion, the rats were lightly re-anesthetized with 1% halothane in 70% nitrous oxide and 30% oxygen, and blood was supplied to the territory of the middle cerebral artery by withdrawing the occluder filament.

2.3. Drug administration

DY-9760e (0.25, 0.50 and 1.00 mg ml^{-1}) was dissolved in 50 mM Sörensen buffer (pH 4.5). Infusion of vehicle (= control) or DY-9760e was started immediately before reperfusion (after 1 h of middle cerebral artery occlusion) at 0.25, 0.50 and 1.00 mg kg^{-1} h^{-1} using an infusion pump (STC-531; Terumo, Tokyo, Japan) for 6 h via a tail vein.

2.4. Physiological parameters

The rats were anesthetized with 2% halothane in 70% nitrous oxide and 30% oxygen. A polyethylene catheter was inserted into a femoral artery for blood pressure recording and blood sampling. Occlusion of the left middle cerebral artery was performed as mentioned above, and then the anesthesia was discontinued. Six rats each were treated with vehicle or DY-9760e (1.00 mg kg^{-1} h^{-1} for 6 h). Rectal temperature (BAT-12/RET-2; Sensortek, NJ, USA), mean blood pressure (AP-621G/RT-1200/DX-100; Nihon Kohden, Tokyo, Japan), arterial blood gases and pH (Blood Gas System M280; Chiron, CA, USA) were measured after 6 h of middle cerebral artery reperfusion.

2.5. Infarct volume

Eleven to fifteen rats were treated with vehicle or doses of DY-9760e. After 24 h of middle cerebral artery reperfusion, the rats were killed by decapitation. The brain was removed and chilled in ice-cold saline for 10 min. Eight 2-mm coronal slices were cut with a brain slicer, beginning from the anterior pole, and the slices were immersed in a saline solution containing 2% 2,3,5-triphenyltetrazolium chloride (Wako, Osaka, Japan) at 37°C for 30 min and fixed by immersion in 10% formalin neutral buffer solution (pH 7.4, Wako) for 1 h. Each brain slice was photographed with a digital camera (DC-1, Ricoh, Tokyo, Japan).

Table 1
Effect of DY-9760e on physiological parameters in the transient middle cerebral artery-occluded rats

	Vehicle	DY-9760e
pH	7.45 ± 0.01	7.45 ± 0.01
Rectal temp. (°C)	37.9 ± 0.2	37.8 ± 0.2
PO_2 (mm Hg)	88.9 ± 2.0	90.0 ± 1.9
PCO_2 (mm Hg)	35.1 ± 1.1	33.5 ± 1.0
MABP (mm Hg)	125 ± 2	121 ± 6

The transient occlusion of the left middle cerebral artery was performed as described in Section 2. DY-9760e was infused at 1.00 mg kg^{-1} h^{-1}. Six rats each were treated with vehicle or DY-9760e. All parameters were measured after 6 h of reperfusion under the conscious condition. Student's *t*-test was used to compare the physiological parameters between the experimental groups. DY-9760e did not significantly change any of the parameters measured.

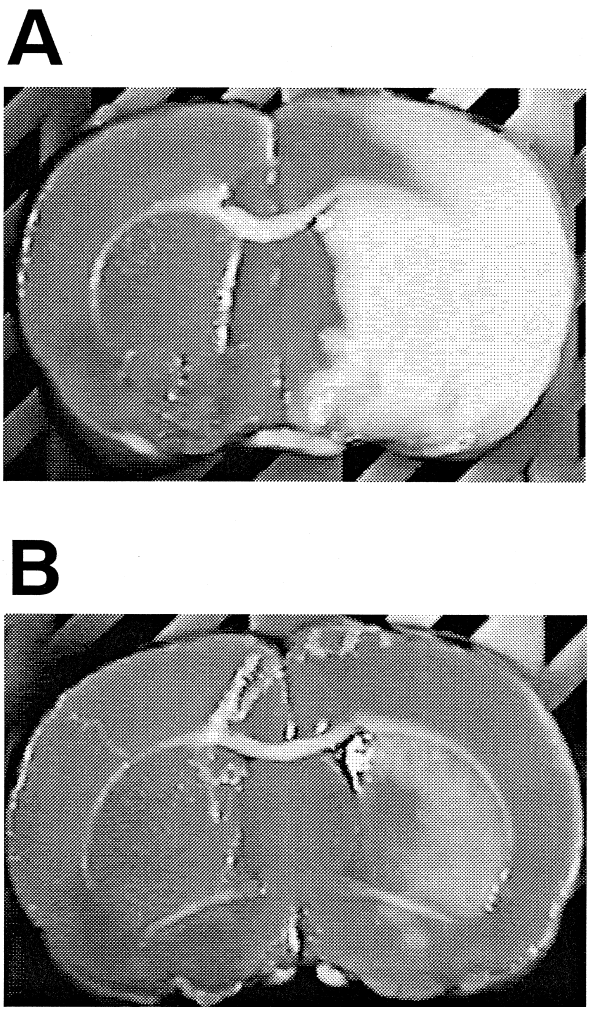

Fig. 1. Representative 2,3,5-triphenyltetrazolium chloride-stained coronal brain sections from rats subjected to transient middle cerebral artery occlusion. Rats were treated with vehicle (A) or DY-9760e at a dose of 1.00 mg kg^{-1} h^{-1} for 6 h (B) from 1 h after middle cerebral artery occlusion.

The infarct area in each slice was blindly evaluated from the saved digital image with an image analyzing system (Quantimet-600, Leica Cambridge, Cambridge, UK). The total infarct volume was determined by summing the infarct volumes of the 8 slices (the infarct volume of each slice was obtained by multiplying the infarct area by 2 mm, the slice thickness).

2.6. Degradation of fodrin

Five rats were treated in each experimental group. At 24 h after middle cerebral artery reperfusion, the cortical and striatal tissues of each hemisphere were separated, and then each tissue was quickly frozen in powdered dry ice. Samples were homogenized in ice-cold buffer containing 50 mM Tris–HCl (pH 7.5), 0.1 mM luepeptin (Funakoshi, Tokyo, Japan), 0.1 mM phenylmethylsulfonyl fluoride (Sigma, MO, USA) and 10 μg ml^{-1} of aprotinin (Sigma). Each homogenate was diluted 1:1 in loading buffer (Daiichi Pure Chem., Tokyo, Japan) containing 62.5 mM Tris–HCl (pH 6.8), 5% 2-mercaptoethanol, 2% sodium dodecyl sulfate (SDS) and 10% glycerol, and boiled for 5 min. The samples containing 20 μg of protein were separated by polyacrylamide gel electrophoresis in the presence of 7.5% (w/v) SDS, and transferred to nitrocellulose using Western blotting. A self-made polyclonal antibody to detect the breakdown products (150-kDa) of α-fodrin (240-kDa) after proteolysis by calpain was made in accordance with the procedure described by Roberts-Lewis et al. (1994). In brief, the peptide CQQEVY, corresponding to the COOH-terminus of the NH_2-terminal α-fodrin fragment, was synthesized and conjugated to keyhole limpet hemocyanin

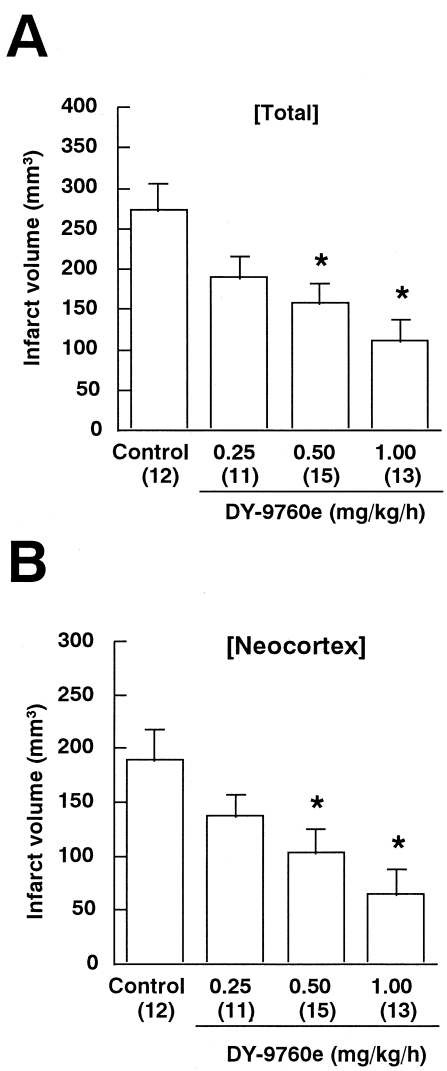

Fig. 2. Effects of DY-9760e on cerebral infarct volume in transiently middle cerebral artery-occluded rats. (A) Effect on hemispheric total infarct volume, and (B) effect on cortical infarct volume. Rats were treated with vehicle or DY-9760e at a dose of 0.25, 0.50 or 1.00 mg kg^{-1} h^{-1} for 6 h from 1 h after middle cerebral artery occlusion. The number of animals in each experimental group is given in parentheses. ANOVA followed by Dunnett's test was used to compare the infarct volume among the experimental groups. *, $P < 0.05$ versus control.

using an Imject® activated immunogen conjugation kit (Pierce, IL, USA). This conjugate was used to immunize rabbits. We obtained an affinity-purified polyclonal antibody. We confirmed that the antibody (1:1500), did not react with 240-kDa α-subunit fodrin and that it specifically recognized the 150-kDa breakdown products produced by calpain proteolysis. The immunoreactive species were detected with a chemiluminescent detection system (ECL, Amersham International, Buckinghamshire, UK). The band of interest was quantified by using a laser scanning densitometer (CS-9000, Shimadzu, Kyoto, Japan). The amount of fodrin breakdown products in each sample is expressed as a percentage of the mean ipsilateral level in the vehicle-treated group. The amount of protein in samples was determined using BCA™ protein assay reagent (Pierce) with albumin as standard.

2.7. Statistical analysis

All data are expressed as means ± S.E.M. Student's *t*-test was used to compare the physiological parameters between the vehicle- and DY-9760e-treated groups. One-way analysis of variance (ANOVA) followed by Dunnett's test was used to compare the infarct volume (or area) and the amount of fodrin breakdown products among the experimental groups. *P*-values less than 0.05 were considered significant.

3. Results

3.1. Effects of DY-9760e on physiological parameters

Table 1 gives a summary of physiological parameters measured at the end of a continuous infusion for 6 h. DY-9760e (1.00 mg kg^{-1} h^{-1}) did not produce any changes in pH, rectal temperature, blood gasses or mean blood pressure.

3.2. Effect of DY-9760e on cerebral infarct volume

The middle cerebral artery occlusion caused infarction in the middle cerebral artery territory including the entire striatum and the dorsolateral and lateral portions of neocortex, but rarely involved the frontal portion of the neocortex or part of the thalamus and hippocampus (Fig. 1A). DY-9760e significantly reduced the total volume of infarction, in a dose-dependent manner, when infused at 0.50 or 1.00 mg kg^{-1} h^{-1} for 6 h starting 1 h after the middle cerebral artery occlusion (Fig. 2A). The reduction was pronounced in the neocortex (Figs. 1B and 2B). Infarct areas in the eight coronal sections are presented in Fig. 3. Statistically significant decreases in infarct area were detected in slice Nos. 2–5 at the dose of 0.50 mg kg^{-1} h^{-1} and slice Nos. 2–6 at that of 1.00 mg kg^{-1} h^{-1}.

3.3. Effect of DY-9760e on fodrin breakdown

It is known that calpain-mediated proteolysis of fodrin yields stable cleavage products of approximately 150-kDa (Seubert et al., 1987; Johnson et al., 1991; Saido et al., 1993; Roberts-Lewis et al., 1994; Bartus et al., 1995). We confirmed that 150-kDa bands of fodrin breakdown products produced by calpain were detected in homogenates of rat brain with the antibody described in Section 2 and that a good linearity was obtained for quantitative blots in the ranges of 5–25 μg of protein (data not shown).

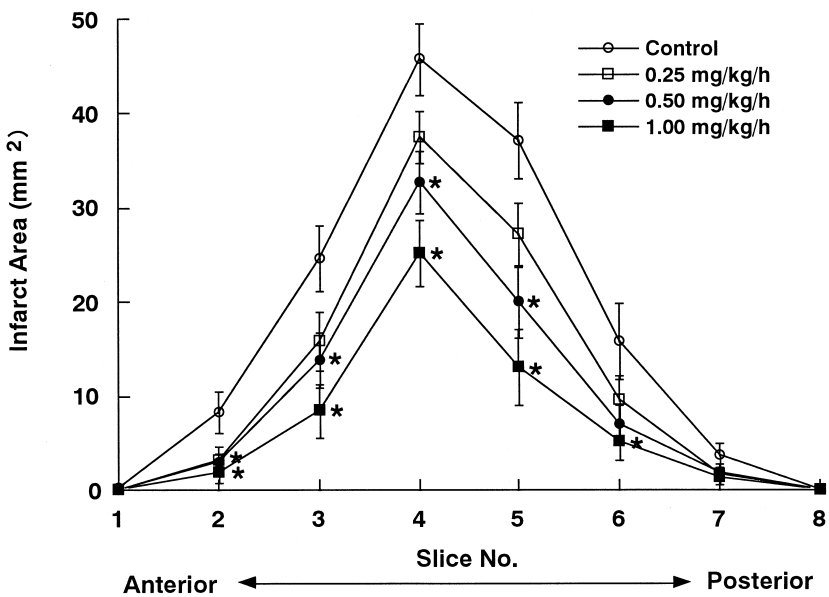

Fig. 3. Effects of DY-9760e on cerebral infarct area of each coronal section in transiently middle cerebral artery-occluded rats. ANOVA followed by Dunnett's test was used to compare the infarct area among the experimental groups. *, $P < 0.05$ versus control.

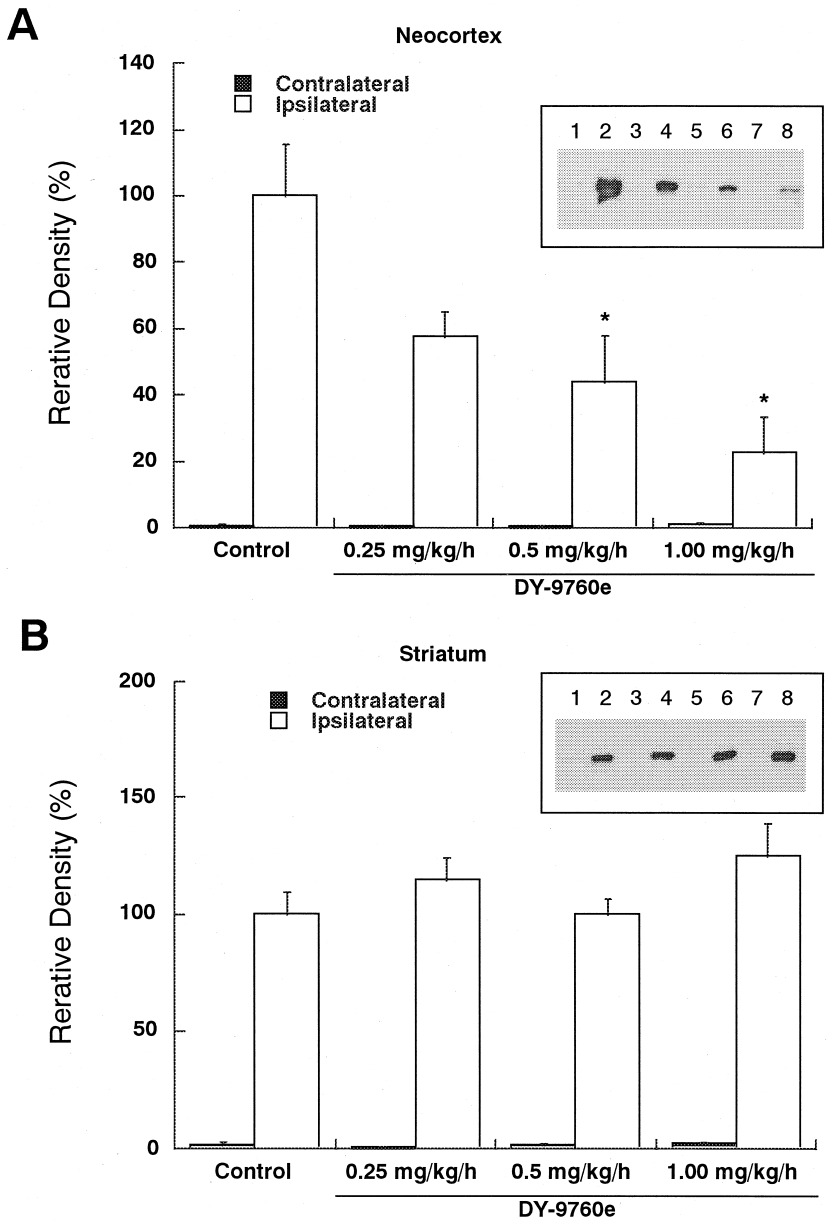

Fig. 4. Effects of DY-9760e on fodrin breakdown in the neocortex (A) or the striatum (B) of rat following transient middle cerebral artery occlusion. Rats ($n = 5$, in each group) were treated with vehicle or DY-9760e at a dose of 0.25, 0.50 or 1.00 mg kg^{-1} h^{-1} for 6 h from 1 h after middle cerebral artery occlusion. Fodrin breakdown products at 24 h after middle cerebral artery reperfusion were measured by Western blot using a specific antibody and densitometric analysis. ANOVA followed by Dunnett's test was used to compare the peak area value of fodrin breakdown products among the experimental groups in each hemisphere. $*$, $P < 0.05$ versus control. The insets represent Western blots using homogenates of equal amounts of protein. Lanes 1,3,5 and 7: contralateral hemisphere treated with vehicle or DY-9760e at a dose of 0.25, 0.50 or 1.00 mg kg^{-1} h^{-1}, respectively; and lanes 2,4,6 and 8: in the ipsilateral hemisphere treated with vehicle or DY-9760e at a dose of 0.25, 0.50 or 1.00 mg kg^{-1} h^{-1}, respectively.

The breakdown products of fodrin were analyzed in the brain 24 h after transient focal ischemia. Pronounced immunoreactivity for fodrin breakdown products was detected in both the ipsilateral cortex and the striatum (Fig. 4A and B). Treatment with DY-9760e (0.50 and 1.00 mg kg^{-1} h^{-1} for 6 h) significantly attenuated the breakdown of fodrin in the cortex (Fig. 4A) in a dose-dependent manner, while DY-9760e was not effective in the striatum (Fig. 4B). In the contralateral hemisphere, little immunoreactivity for fodrin breakdown products was detected in either the cortex or the striatum in any experimental group (Fig. 4A and B).

4. Discussion

We previously reported that DY-9760e, a potent and novel calmodulin antagonist, had a cytoprotective effect against cell death induced by Ca^{2+} ionophore in neuroblastoma cells (Sugimura et al., 1997). The present study

indicates that DY-9760e can reduce infarct volume and the breakdown of fodrin in transiently middle cerebral artery-occluded rats.

It has been widely accepted that an excessive elevation of the intracellular Ca^{2+} concentration during ischemia is a trigger to neuronal damage (Choi, 1988, 1995; Meldrum and Garthwaite, 1990; Mitani et al., 1993). Such an elevation of the neuronal Ca^{2+} concentration may lead to aberrant activation of Ca^{2+}/calmodulin-dependent pathways, such as NO synthase (Nagafuji et al., 1992; Buisson et al., 1993; Dawson et al., 1993b), calcineurin (Dawson et al., 1993a; Sharkey and Butcher, 1994; Ide et al., 1996) and Ca^{2+}/calmodulin-dependent protein kinase II (Hajimohammadreza et al., 1995), which appear to mediate cell death in neurons. Recent studies have shown that 7-nitroindazole, which is a neuronal NO synthase inhibitor, or tacrolimus (FK506), which is a calcineurin inhibitor, reduces infarct volume in a focal ischemic model (Yoshida et al., 1994; Sharkey et al., 1996).

Fodrin is known to be a preferential substrate for calpain (Siman et al., 1984). Bartus et al. (1995) have shown that the breakdown of fodrin increased time-dependently without the change in the native fodrin content after cerebral ischemia, and that fodrin breakdown induced by ischemic insults precedes neuronal death, which suggest that fodrin breakdown products are a valuable marker for neuropathological events. Interestingly, it has previously been shown that calmodulin markedly stimulates the degradation of fodrin by calpain (Seubert et al., 1987; Johnson et al., 1991). DY-9760e reduced, in a dose-dependent manner, fodrin breakdown in the ipsilateral cortex (Fig. 4), but did not affect calpain I and II activities at concentrations up to 100 μM (Sugimura et al., 1997). This suggests that the reduction of fodrin breakdown by DY-9760e is due at least in part to its calmodulin-antagonistic action. DY-9760e failed to reduce fodrin breakdown in the ipsilateral striatum, indicating a rapid onset of fodrin breakdown in this region. In fact, it is widely accepted that the striatum corresponds to the ischemic core, while the neocortex lies perifocally, which is called 'the penumbra' (Astrup et al., 1981; Memezawa et al., 1992). Furthermore, magnetic resonance imaging has shown that the development of cerebral infarction in the striatum precedes that in the neocortex in middle cerebral artery-occluded rats (Gill et al., 1995; Hoehn-Berlage, 1995; Reith et al., 1995) and that fodrin in the striatum is more rapidly degraded than that in the cortex (Bartus et al., 1995; Takagaki et al., 1997).

Since DY-9760e had no significant influence on cerebral blood flow in spontaneously hypertensive rats subjected to bilateral carotid artery ligation, nor in untreated Wistar rats (data not shown), it is likely that its cerebroprotective effect is attributable to a direct action in the cerebral parenchyma. Further investigation is needed to evaluate the mechanism by which DY-9760e exerts its cerebroprotective effects. Interestingly, a recent study shows that trifluoperazine, a calmodulin antagonist, reduces infarct volume in transient middle cerebral artery-occluded rats (Kuroda et al., 1997). Although Kuroda et al. (1997) have concluded that trifluoperazine alone does not qualify as an important therapeutic agent because of its narrow therapeutic window, this problem may be due to its weak calmodulin-antagonistic activity. In fact, we confirmed that the K_i values of DY-9760e and trifluoperazine for calcineurin were 2.0 and 27.6 μM, respectively (personal data). Our findings suggest that calmodulin plays a critical role in the processes of ischemic brain injury.

In conclusion, DY-9760e reduced infarct volume and fodrin breakdown in transiently middle cerebral artery-occluded rats. This compound may have a therapeutic effect in the acute phase of cerebral ischemic damage.

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