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Neuroprotective Efficacy of YM872, an α-Amino-3-Hydroxy-5-Methylisoxazole-4-Propionic Acid Receptor Antagonist, after Permanent Middle Cerebral Artery Occlusion in Rats

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ABSTRACT

The neuroprotective efficacy of YM872, a novel, highly water-soluble α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor antagonist, was investigated in rats subjected to permanent occlusion of the left middle cerebral artery. The rats were assessed either histologically or neurologically 24 hr or 1 wk after ischemia. YM872 was intravenously infused for either 4 or 24 hr at dose rates of 0 to 20 mg/kg/hr starting 5 min after ischemia to examine the effect of prolonged treatment. YM872 was then infused at 20 mg/kg/hr beginning 0 to 4 hr after ischemia to determine the efficacy time window. Additionally, a 20 mg/kg/hr dose rate of YM872 was infused for 4 hr in single day- or 5-day repetitive-administrations to evaluate long-term benefits of the drug. YM872 significantly reduced infarct volume in both 4- and 24-hr treatment groups measured 24 hr after ischemia. No difference was observed in the degree of protection between length of infusion. Significant neuroprotection was maintained even when drug administration was delayed up to 2 hr after ischemia. A single YM872-administration significantly improved neurological deficit and reduced infarct volume (30%, $P < .01$) measured 1 wk after ischemia. YM872 treatment did not induce such adverse effects as physiological changes, serious behavioral abnormalities or nephrotoxicity. These data suggest that the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor plays a crucial role in the progression of neuronal damage in the early phase of ischemia and that YM872 may be useful in treating acute ischemic stroke.

The involvement of the AMPA glutamate receptor, as well as the NMDA glutamate receptor, in the development of ischemia-induced neuronal damage has been previously demonstrated (Gill, 1994). First generation antagonists of the AMPA receptor, quinoxalinedione derivatives such as NBQX, have been shown to be neuroprotective against neuronal damage following focal cerebral ischemia (Gill, 1994; Graham *et al.*, 1996; Yatsugi *et al.*, 1996). However, the poor water solubility and resultant renal toxicity of these compounds may have limited their experimental and clinical usefulness (Xue *et al.*, 1994). Improving the solubility of AMPA antagonists is thus an important matter to assess their suitability as neuroprotective agents.

Recently, the novel competitive AMPA receptor antagonist YM872 was discovered as a highly water-soluble agent keeping the selectivity and potency for AMPA receptors (K_i = 0.096 μM) (Kohara *et al.*, 1996; Takahashi *et al.*, 1998). The solubility of YM872 in pH 7 buffers is approximately 800 times greater than NBQX and YM90K, which are also competitive AMPA antagonists (Kohara *et al.*, 1996). It has been shown that constant intravenous infusion of YM872 produced significant protection against neuronal damage induced by focal cerebral ischemia in anesthetized cats (Takahashi *et al.*, 1998).

Although it is well established that AMPA antagonists protect against ischemic neuronal damage in a range of animal focal ischemia models, the optimal duration of treatment with AMPA antagonists remains unresolved. Because of this, there is a possibility that early termination of drug administration may reduce its beneficial effects against ischemic damage. In addition, few studies have assessed the maximum time that initiation of drug administration could be delayed following the onset of ischemia although still maintaining significant protection (Xue *et al.*, 1994; Graham *et al.*, 1996). This interval between the onset of ischemia and drug administration is critical in defining the potential clinical utility of any neuroprotective agent.

Focal cerebral ischemia induced by unilateral permanent

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ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; ANOVA, analysis of variance; MABP, mean arterial blood pressure; MCA, middle cerebral artery; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo (f) quinoxaline; NMDA, N-methyl-D-aspartate; TTC, 2,3,5-triphenyltetrazolium hydrochloride; VSCC, voltage-sensitive calcium channel; YM90K, 6-(1*H*-imidazol-1-yl)-7-nitro-2,3(1*H*, 4*H*)-quinoxalinedione monohydrochloride; $[Ca^{++}]_i$, intracellular Ca^{++} concentration.

occlusion of the MCA in animals is the most relevant model of human stroke presently available (Graham, 1988). This experimental model in rats, first described by Tamura *et al.* (1981), is widely used to determine the therapeutic potential of putative neuroprotective agents. In our study, therefore, this model was used to investigate the neuroprotective properties of YM872 when administered by constant intravenous infusion, which is the intended mode of administration for clinical use in treating acute ischemic stroke. The end-points measured were either infarct volume or neurological status 24 hr or 1 wk after ischemia.

Methods

Animal Preparation

The experimental procedures were performed in accordance with the guidelines of the Animal Ethical Committee of Yamanouchi Pharmaceutical Co., Ltd. Male Fischer 344 rats (Charles River Japan, Yokohama, Japan) weighing 280 to 350 g were used. The animals were maintained on ordinary laboratory chow and tap water at libitum under a constant 12-hr light-dark cycle.

The rats were anesthetized with halothane in a mixture of 25% O_2/75% N_2 delivered through a close-fitting facial mask. Anesthesia was induced with 4% halothane and subsequently maintained with 0.5 to 1.5% halothane. Rectal temperature was monitored using a rectal probe and maintained around 37°C using a thermostatically controlled heating blanket and an overhead lamp (ATB-1100, Nihon Kohden, Tokyo, Japan) during the operation. For constant intravenous infusion of saline or YM872, a polyethylene tube 50 (PE-50) was inserted into the left jugular vein, externalized at the dorsal neck and covered with tape until drug administration. The rats were placed under an operating microscope on their right sides. The proximal portion of the left MCA was then permanently occluded according to the method described by Tamura *et al.* (1981). Briefly, through a 2-cm skin incision, the temporal muscle was retracted via a trans-retro-orbital approach without removal of the temporal muscle or zygomatic arch. A small subtemporal craniectomy was performed, and the dura was incised with a sharp needle. Cerebral ischemia was induced by microbipolar coagulation using forceps connected to an electrosurgical unit (MC-1, Keisei Ika Kogyo Co., Ltd., Tokyo, Japan). The occlusion was made at the stem of the MCA just medial to the olfactory tract. The MCA was then transected to ensure completeness of occlusion. The time of transection was taken as the exact time of MCA occlusion. The craniectomy wound was then sutured and the anesthesia was turned off. Body temperature was maintained with heating lamps until the animals were fully conscious. In the dosing regimen used in our experiments, YM872 had no apparent effects on the rats in terms of mortality or recovery from the anesthesia. No animals died during the study, and all rats awakened from the halothane anesthesia within 20 min after halothane was discontinued.

Physiological variables, including brain temperature, were measured in eight of these rats divided into two groups: one treated with saline (10 ml/kg/hr for 24 hr; $n = 4$) and the other with the high dose of YM872 (20 ml/kg/hr in a dose volume of 10 ml/kg/hr for 24 hr; $n = 4$). Four days before induction of ischemia, a guide cannula for a needle-type thermoprobe (BAT-12, Physitemp Instrument Inc., Clifton, NJ) was implanted under pentobarbital anesthesia (50 mg/kg, i.p.) into the left striatum (A: 0.0 mm, L: 2.5 mm, H: 3.5 mm from bregma) of these rats to monitor brain temperature. The guide cannula was carefully implanted as to minimize the surgical damage, so that the infarct volume was not affected by this procedure. On the day of surgery for MCA occlusion, a PE-50 was introduced into the tail artery of these rats under halothane anesthesia for blood pressure monitoring and blood sample analysis. The variables were obtained 30 min before MCA occlusion while the anesthesia was briefly turned off and 4, 12 and 24 hr after MCA occlusion when the rats were conscious.

Experimental Protocol

Infusion system. The experimental protocol is schematized in figure 1. After surgery the animals were put in spacious cages for drug administration. In our study the drug or saline was administered by constant i.v. infusion. Preliminary experiments (Kayama *et al.*, unpublished data) indicated that the plasma concentration of YM872 reached a near-steady state 1 hr after the start of infusion and was eliminated rapidly after the termination of infusion in intact rats (plasma half-life: 0.12 hr in distribution phase and 1.50 hr in terminal phase). The infusion was conducted according to a previously described method (Shintani *et al.*, 1993; Nakaki *et al.*, 1981). The tip of the cannula exiting from the dorsal neck was connected to a cannula swivel device (375/23, Instech Laboratories, Inc., PA) attached to the roof of the spacious cage. This device was used to allow bi-directional rotation while the drug was administered. A PE-50 extending from the other end of the device was joined to a disposable syringe which was fixed to an infusion apparatus (STC-525, Terumo, Tokyo, Japan). This tethering system allowed the rats free movement without interference with the patency of the cannula.

Twenty four-hour evaluation. The effect of YM872 on infarct volume was assessed 24 hr after the onset of ischemia. In the extended infusion study (fig. 1a), two different dosing regimens, 4- or 24-hr infusion, were chosen to investigate the neuroprotective effect of prolonged YM872 treatment duration. Prior studies reported that glucose metabolism is irreversibly lost throughout the MCA distri-

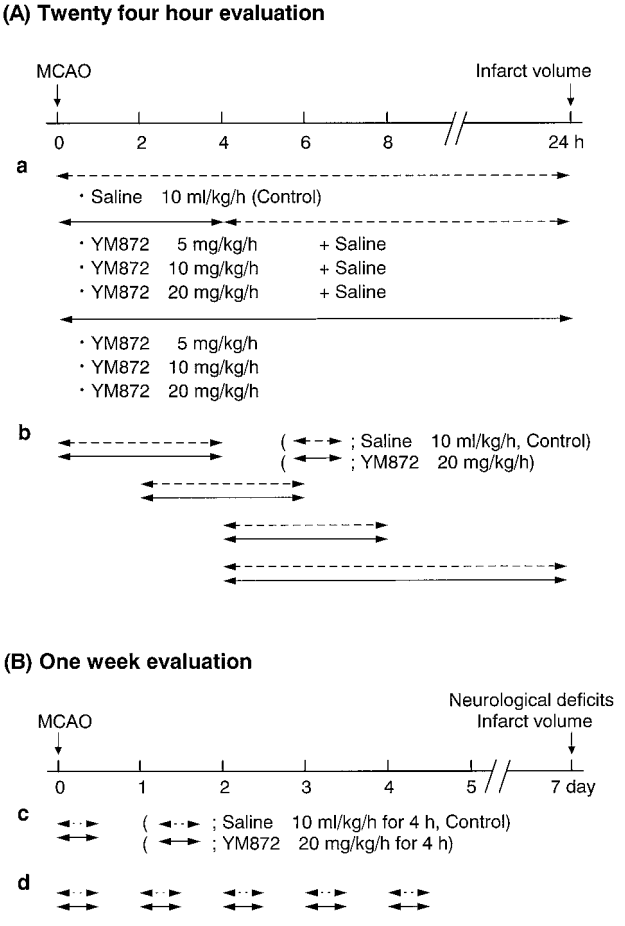

Fig. 1. Experimental protocols. A, 24-hr evaluation study of (a) the extended infusion study and (b) the time window study. B, 1-wk evaluation study of (c) a single administration and (d) a 5-day administration. MCAO, Middle cerebral artery occlusion.

bution (Shiraishi *et al.*, 1989) and ischemic damage is thought to occur (Nedergaard, 1988) within 4 hr after the onset of ischemia in this permanent MCA occlusion model. A 24-hr treatment was performed to cover a survival period, during which the tissue injury develops into an infarct and the ischemic lesion reaches its maximum size (Gill *et al.*, 1992; Takasago *et al.*, 1997). YM872 (MW 349.3; Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan) was dissolved in isotonic saline alkalized with a few drops of 1 N sodium hydroxide (NaOH) solution to adjust the pH to 7.4. The animals were divided into seven treatment groups. Assignment of an animal to either the saline or the YM872 treatment groups was performed in a random manner. YM872 was i.v. infused at a rate of 5, 10 or 20 mg/kg/hr in a dose volume of 10 ml/kg/hr starting 5 min after the onset of ischemia, and was continued for either 4 hr of YM872 followed by a 20-hr infusion of saline (pH 7.4) at a rate of 10 ml/kg/hr (n = 10 in each group) or 24 hr (n = 8, 8 or 10 respectively). Control animals were given an intravenous infusion of saline (pH 7.4) at a rate of 10 ml/kg/hr for 24 hr (n = 12). In this study, physiological variables were measured in two groups of rats, those treated with saline (control; n = 4) and those with the high dose of YM872 (20 ml/kg/hr for 24 hr; n = 4).

YM872 was then infused i.v. to separate groups of rats in a delayed fashion at a rate of 20 mg/kg/hr over 4 hr to determine the time window of efficacy (fig. 1b). Five-minute, 2-hr or 4-hr delay periods after MCA occlusion were studied (n = 8 in each group). In control rats, infusion of saline (10 ml/kg/hr for 4 hr) was also begun 5 min, 2 hr or 4 hr after MCA occlusion (n = 8 in each group). In a further experiment (fig. 2b), YM872 was infused at a rate of 20 mg/kg/hr over 20 hr starting 4 hr after the onset of ischemia (n = 8). Controls for this group were given an intravenous infusion of saline (10 ml/kg/hr) over 20 hr (n = 8).

During drug administration, behavioral changes such as stereotyped behavior were checked. The animals were kept normothermic using an overhead heating lamp throughout the survival period of 24 hr. Food and water were allowed *at libitum*.

One week evaluation. To exclude the possibility that the AMPA antagonist might simply delay the evolution of ischemic damage, the effects of YM872 on neurological status and infarct volume were assessed after 1 wk of survival. Two dosing regimens were evaluated to assess the benefit of repetitive treatment of YM872 (fig. 1c and d). In one group (fig. 1c), YM872 was intravenously infused at a rate of 20 mg/kg/hr in a dose volume of 10 ml/kg/h starting 5 min after the onset of ischemia and was continued for 4 hr (n = 8) (single-administration group). In another group (fig. 1d), YM872 at a dose rate of 20 mg/kg/hr was intravenously infused for 4 hr starting 5 min after ischemia and was then infused for 4 hr once daily over the following 4 days (n = 8) (5-day administration group). Control animals for these groups (n = 8 in each group) were given an intravenous infusion of saline (pH 7.4) at a rate of 10 ml/kg/hr according to the respective YM872-treatments. During drug administration, the rats were kept normothermic using an overhead heating lamp, and behavioral changes were checked. After each treatment the rats were returned to the animal house and were allowed free access to food and water.

One week after MCA occlusion, the neurological status of the rats was assessed in a blind fashion. The rating scales for neurological deficit were used in accordance with the method of Bederson *et al.* (1986) with some modifications. The following were assessed: 1) the degree of spontaneous activity, 2) right forepaw hemiplegia, 3) failure to extend right forepaw when the rat was lifted by its tail, 4) resistance to lateral push, 5) inclined posture to the right, 6) circling to the right and 7) response to vibrissae touch. Each sign was scored using the following criteria: grade 0, no abnormality; grade 1, mild abnormality; grade 2, severe abnormality. The scores on each item were summed into a total with the lowest possible score of 0 and a highest possible score of 14.

Histological Quantification

Infarct measurement 24 hr after MCA occlusion. At 24 hr after MCA occlusion, rats were anesthetized with pentobarbital sodium (50 mg/kg i.p.) and decapitated. The kidneys of the rats were removed to check for precipitation in the segment of the kidney. The cerebrum was rapidly removed and coronally sectioned in 1-mm thick slices from 4.0 mm anterior to 8.0 mm posterior to the bregma using a tissue chopper (Mcilwain Tissue Chopper, The Mickle Laboratory Engineering Co. Ltd., Surrey, UK). Twelve consecutive slices were stained with a 2% solution of TTC (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) and fixed by immersion in 10% phosphate-buffered formalin. The posterior surface of each section was recorded with a CCD color video camera system (ICD-740, Olympus-Ikegami, Tokyo, Japan). Areas not stained with TTC, which were classified as lesioned, were delineated by an observer unaware of the drug treatment for each rat, and measured by a video image analyzing system (NIH Image version 1.57). The infarct volume was calculated by multiplying lesion areas by the slice thickness. The infarct volumes of hemisphere and cortex of the drug-treatment groups were expressed as percentage (the mean ± S.E.) of the mean infarct volume of the respective saline-treated control groups.

Infarct measurement 1 wk after MCA occlusion. After the assessment of neurological status 1 wk after MCA occlusion, rats were anesthetized with pentobarbital sodium (50 mg/kg i.p.). Rats were perfusion-fixed for neuropathological analysis with 4% formaldehyde in 0.1 M phosphate-buffer (pH 7.4) at a pressure equal to the MABP (95–120 mm Hg). The kidneys of the rats were removed to check for precipitation in the segment of the kidney. Rats were decapitated immediately after perfusion fixation, and the heads stored in fixative for at least 24 hr. The brains were then removed. After the hindbrains had been detached, the forebrains were embedded in paraffin wax and sectioned at multiple levels. The sections, stained with hematoxylin-eosin, were examined by light microscopy. Neuronal damage was defined on the basis of the following morphological characteristics: microvacuolation, shrinkage of the neuropil and presence of dark neurons and eosinophilic neurons. The infarct area was delineated at eight preselected coronal levels under microscopic observation by an observer unaware of the drug treatment for each rat. The infarct area was calculated with a computer-aided image analyzer system (Luzex III, Nireco Co., Tokyo, Japan). The infarct volume in each brain was determined by integrating these areas. The endpoints for integration were anterior 2.7 mm and posterior 8.0 mm according to Paxinos and Watson (1986). The hemispheric and cortical infarct volumes of the drug-treatment groups were expressed as percentage (the mean ± S.E.) of the mean infarct volume of their respective saline-treated control groups.

Statistical Analysis

Data, except for neurological deficit scores, are presented as the mean ± S.E. For physiological variables (table 1), statistical comparison between the saline-treated control and the YM872-treated groups was performed by two-way repeated measures ANOVA. For comparing infarct volumes within the four different treatment regimens (fig. 1a–d), statistical significance of differences was estimated using Student's t test with Bonferroni adjustment for multiple comparisons. For neurological deficit scores, the individual values are plotted and the median values are presented (fig. 6). Statistical significance of differences among the four groups was calculated using Steel-Dwass test. Regression analysis was performed to compare the neurological deficit score and the infarct volume assessed 1 week after ischemia. $P < .05$ was considered significant.

Results

Physiological variables and renal toxicity. Physiological variables including blood pressure, pH, blood gases, rectal and brain temperatures for the two groups of rats treated

TABLE 1
Physiological variables measured before and after MCA occlusion in rats

		Pre-MCAO	Post-4 hr	12 hr	24 hr
MABP (mmHg)	Saline	102 ± 11	114 ± 2	115 ± 2	107 ± 8
	YM872	119 ± 5	100 ± 8	100 ± 6	112 ± 8
pH	Saline	7.42 ± 0.01	7.40 ± 0.01	7.39 ± 0.02	7.40 ± 0.02
	YM872	7.42 ± 0.01	7.41 ± 0.01	7.43 ± 0.01	7.45 ± 0.01
Paco2 (mmHg)	Saline	42.6 ± 1.4	38.6 ± 0.5	39.0 ± 1.4	36.3 ± 1.1
	YM872	42.4 ± 0.6	38.8 ± 0.3	37.7 ± 1.1	37.8 ± 0.6
Pao2 (mmHg)	Saline	83.0 ± 2.3	82.3 ± 1.8	87.1 ± 2.3	95.3 ± 2.3
	YM872	96.6 ± 12.1	85.2 ± 1.5	89.1 ± 1.9	88.9 ± 3.0
Hct (%)	Saline	47.8 ± 0.5	43.2 ± 0.9	41.8 ± 0.8	35.3 ± 1.5
	YM872	45.5 ± 0.8	44.0 ± 1.5	42.7 ± 0.6	37.4 ± 0.9
Temperature (°C)					
Rectum	Saline	37.2 ± 0.2	37.7 ± 0.3	37.5 ± 0.1	37.2 ± 0.2
	YM872	37.4 ± 0.1	37.4 ± 0.1	37.3 ± 0.3	37.4 ± 0.2
Brain	Saline	37.3 ± 0.1	37.1 ± 0.2	37.2 ± 0.2	36.9 ± 0.3
	YM872	37.6 ± 0.2	37.5 ± 0.3	37.2 ± 0.3	37.2 ± 0.2

MCAO, Middle cerebral artery occlusion; MABP: mean arterial blood pressure; Hct: hematocrit. Saline (10 ml/kg/hr) or YM872 (20 mg/kg/hr) was infused i.v. starting 5 min after the MCA occlusion and continued for 24 hr. Data are presented as the mean ± S.E. (n = 4 in each group). There are no significant differences between the two groups (two-way repeated measures ANOVA).

with saline and those treated with YM872 (20 mg/kg/hr for 24 hr), in which the infarct volume was significantly reduced (fig. 2), were within normal limits before and after MCA occlusion (table 1). YM872 did not induce any abnormal behavior such as stereotyped behavior during the infusion period. Neither precipitation nor associated tissue damage was observed in the kidneys of YM872-treated animals under light microscopy.

Infarct volumes 24 hr after MCA occlusion. In the extended infusion study (fig. 1a), YM872 dose dependently reduced the hemispheric and cortical infarct volumes when compared to the saline-treated control group (fig. 2). At a rate of 20 mg/kg/hr for 4 hr, YM872 significantly reduced the infarct volume by 23% in the cerebral hemisphere ($P < .01$) and by 26% in the cerebral cortex ($P < .01$) compared with the control group. The neuroprotective efficacy of a 24-hr treatment with YM872 at a rate of 20 mg/kg/hr (27% in the cerebral hemisphere and 31% in the cerebral cortex, $P < .01$ *vs.* control) was slightly better than that observed with a 4-hr treatment. However, there was no significant difference between these two groups.

Similar results were obtained between 4- and 24-hr treatment groups of rats in which 10 mg/kg/hr of YM872 was administered. The degree of protection seen with a 24-hr treatment with YM872 at a rate of 10 mg/kg/hr (19% in the cerebral hemisphere and 22% in the cerebral cortex, $P < .05$ *vs.* control) was same as that produced by a 4-hr treatment with YM872 (19% in the cerebral hemisphere and 20% in the cerebral cortex, $P < .05$ *vs.* control). The 5 mg/kg/hr dose of YM872 did not significantly reduce the hemispheric or cortical infarct volumes in either 4- or 24-hr treatment groups. No dose of YM872 had a significant therapeutic effect on the striatum, the ischemic core area in this model (data not shown).

In the time window study (fig. 1b), YM872, i.v. infused at a rate of 20 mg/kg/hr for 4 hr, significantly reduced the hemispheric and cortical infarct volumes even when the initiation of drug administration was withheld for up to 2 hr after the onset of ischemia (fig. 3). The degree of protection against cortical infarct volume in the 2-hr delayed YM872-treated group was 33% ($P < .05$ *vs.* control), which is similar to that (30%, $P < .01$ *vs.* control) of the 5-min delay treatment group. When started 4 hr after the onset of ischemia, however, no significant differences in the hemispheric or cortical infarct volumes were observed between control and YM872-treated groups. In a further experiment, YM872 was infused at a rate of 20 mg/kg/hr over 20 hr starting 4 hr after the onset of ischemia to investigate whether or not the maximum time for initiation of drug administration could be delayed for 4 hr by extending the duration of subsequent treatment. However, this extended YM872 treatment regimen did not yield significant neuroprotection compared with the saline-treated control group (fig. 3).

Infarct volumes and neurological status 1 wk after MCA occlusion. To ensure that the YM872-treatment prevented, and not simply delayed, neuronal damage after the ischemic insult and to evaluate the benefit of repetitive treatment of YM872, a 1-wk evaluation study was performed.

Single-administration of YM872 at a rate of 20 mg/kg/hr for 4 hr (fig. 1c) significantly reduced the infarct volume by 25% in the cerebral hemisphere ($P < .01$) and by 30% in the cerebral cortex ($P < .01$) compared with the control group (figs. 4 and 5). The neuroprotective efficacy of a 5-day repetitive administration of YM872 (figs. 1d and 5) (28% in the cerebral hemisphere and 36% in the cerebral cortex, $P < .01$ *vs.* control) was almost same as that observed in the single-administration group (fig. 5). Neither regimen of YM872 treatment had a significant therapeutic effect on the striatum (data not shown).

YM872- (20 mg/kg/hr) treated rats of both single- and 5-day administration groups demonstrated significant improvements ($P < .01$) in neurological deficits 1 wk after MCA occlusion compared with the respective control groups (fig. 6). Specifically, YM872-treated animals showed fewer deficits in terms of failure to extend right forepaw, hemiplegia and inclined posture at rest (data not shown). Similar extents of reduction were observed for both single- and 5-day repetitive administrations of YM872.

Regression analysis showed that there was a significant correlation between the neurological deficit score and the infarct volume assessed 1 wk after ischemia for both single- ($r = 0.78$, $P < .01$) and 5-day ($r = 0.72$, $P < .01$) administration groups.

These results indicate that YM872 was not merely postponing but preventing neuronal death after ischemic insult

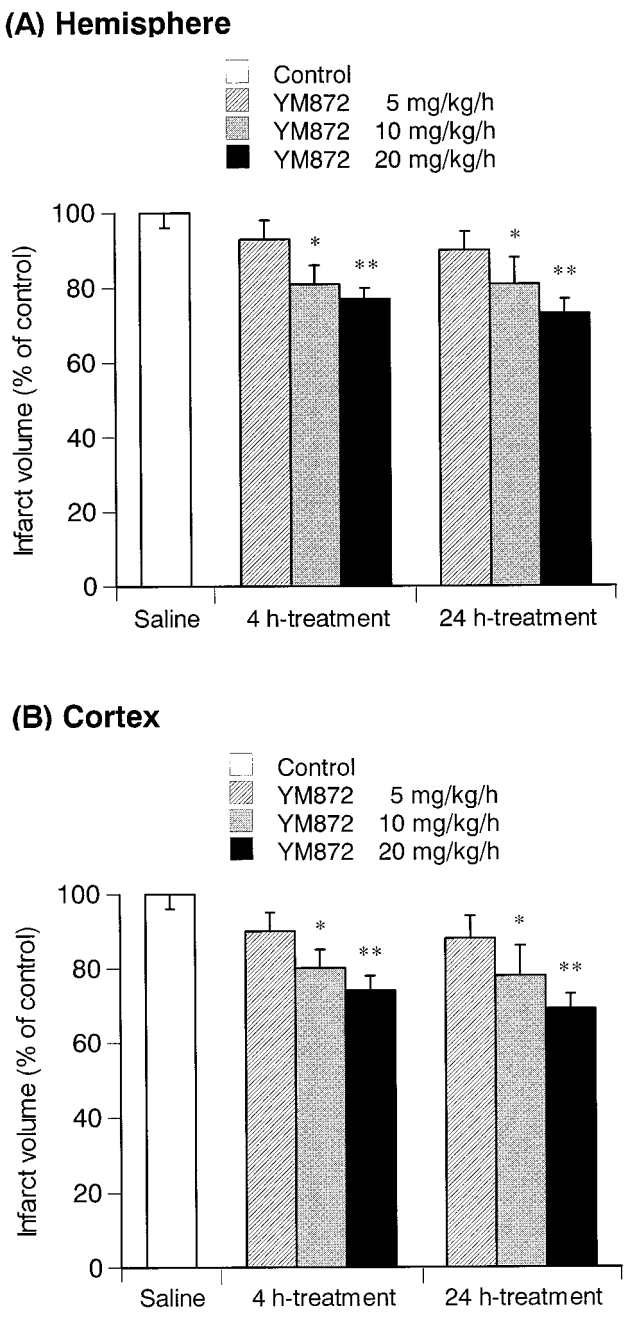

Fig. 2. Neuroprotective effect of YM872 on the volume of ischemic damage in (A) the cerebral hemisphere and (B) the cerebral cortex 24 hr after MCA occlusion in rats. YM872 was infused i.v. starting 5 min after the onset of ischemia, and continued for either 4 hr of YM872 followed by a 20-hr infusion of saline, or 24 hr. Control animals were given an intravenous infusion of saline for 24 hr. Each value represents the mean ± S.E. (n = 12 in saline-treated control group; n = 10 in each YM872, 4-hr treatment group; n = 8 in YM872, 5 or 10 mg/kg/hr, 24-hr treatment group; n = 10 in YM872, 20 mg/kg/hr, 24-hr treatment group). * $P < .05$, ** $P < .01$ *vs.* control. No significant difference was observed in the degree of protection afforded by 4 and 24 hr infusions at each dose.

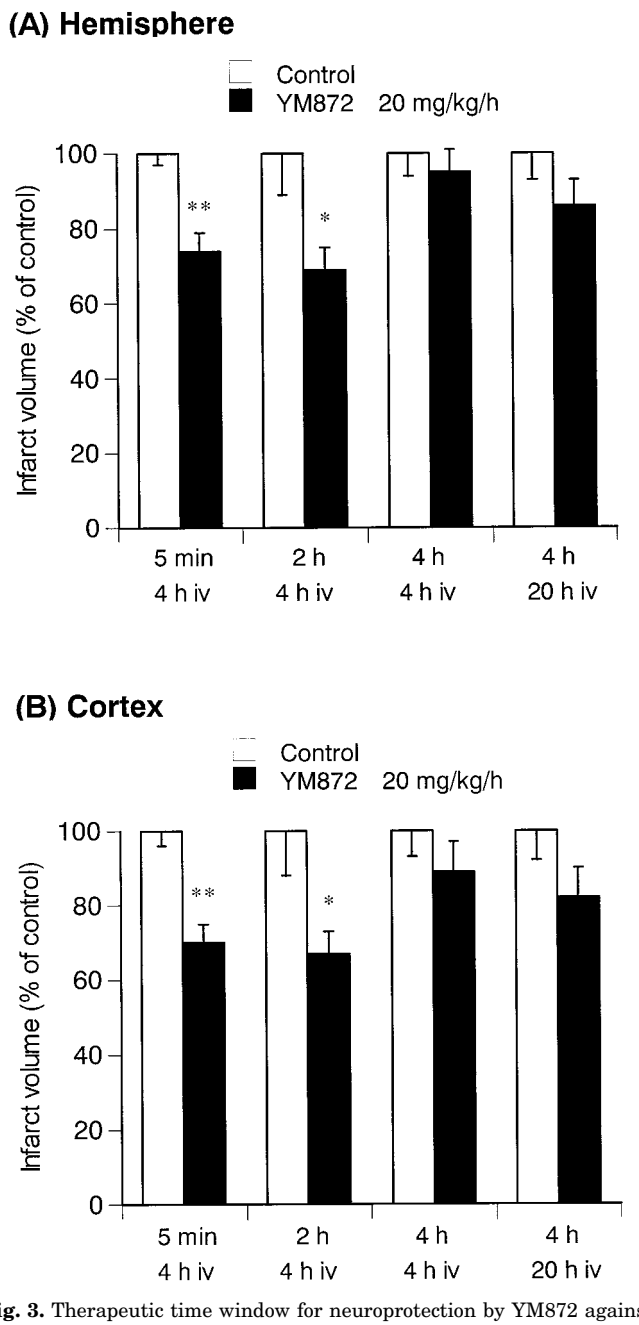

Fig. 3. Therapeutic time window for neuroprotection by YM872 against the volume of ischemic damage in (A) the cerebral hemisphere and (B) the cerebral cortex 24 hr after MCA occlusion in rats. Saline or YM872 (20 mg/kg/h) was intravenously infused starting 5 min, 2 h, or 4 h after the onset of ischemia and continued for 4 hr (5 min, 4 hr i.v.; 2, 4 hr i.v.; 4, 4 hr i.v., respectively). In a further experiment, saline or YM872 (20 mg/kg/hr) was infused over 20 hr starting 4 hr after the onset of ischemia (4 hr, 20 hr i.v.). Each value represents the mean ± S.E. (n = 8 in each group). * $P < .05$, ** $P < .01$ *vs.* respective controls.

and that histological neuroprotection was correlated with functional improvement as well.

Discussion

Our findings demonstrate three marked neuroprotective properties of YM872. First, no significant difference was observed in the degree of protection between durations of infusion for each dose. Second, significant neuroprotection was maintained even when drug administration was delayed up to 2 hr after ischemia. Third, significant improvement in neurological deficit and reduction in infarct volume were observed 1 wk after ischemia after only a single 4-hr administration of YM872. Furthermore, under these dosing regimens, the administration of YM872 did not induce such adverse effects as physiological changes, serious behavioral

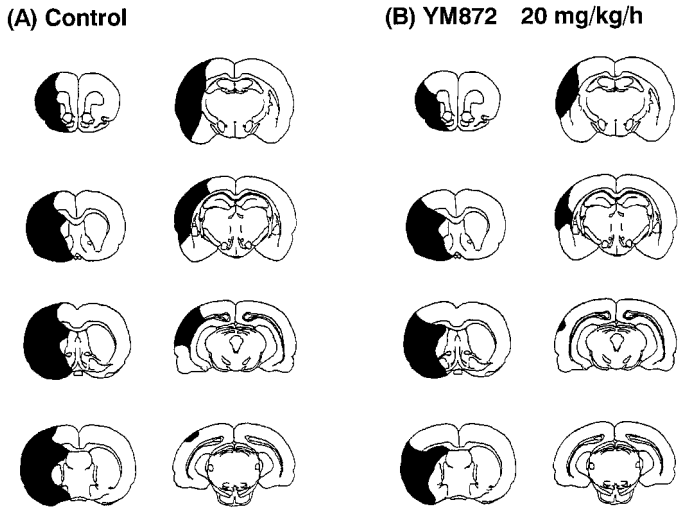

Fig. 4. Areas of ischemic brain damage, as assessed using hematoxylin-eosin, which were used for volumetric analysis one week after MCA occlusion in rats (single-administration group). The area of damage is charted on eight coronal sections representing predetermined stereotaxic levels anterior 2.7 mm to posterior 8.0 mm according to Paxinos and Watson (1986). A, Shows the area of ischemic damage in a saline-treated control rat. Infarction was seen in the dorsolateral cortex and striatum. B, Illustrates the neuroprotective effect of YM872 against cortical but not striatal ischemic damage. Saline or YM872 (20 mg/kg/hr) was i.v. infused for 4 hr starting 5 min after the onset of ischemia.

abnormalities or nephrotoxicity. Although substantial evidence is now available that AMPA antagonists reduce infarct size in various animal models of experimentally induced stroke, only a limited number of studies have assessed either the effects of different treatment durations or the critical time window of efficacy after ischemic insult. Indeed, in the experiments with prototype AMPA antagonists such as NBQX and YM90K, these issues could not be fully addressed because of their insufficient water solubility and resultant high nephrotoxicity potential. YM872, an N1 carboxymethylated derivative of YM90K, is a significantly more water-soluble compound than either YM90K or NBQX (Kohara *et al.*, 1996), and in our study was shown to have the equipotent neuroprotective effect (approximately 30%) to these compounds (Gill *et al.*, 1992; Shimizu-Sasamata *et al.*, 1996) at doses where no precipitation in the kidney was observed. In our study, the neuroprotective effect of YM872 was extensively assessed by employing more flexible dosing regimens made possible by this increased solubility.

In terms of the neuroprotective effects of prolonged YM872 treatment duration, no difference was observed in the degree of protection afforded by 4 or 24 hr infusions. This indicates that the neuroprotective efficacy of YM872 was not enhanced by extending the treatment duration. Similar findings regarding the extent of improvement in neurological deficit and the degree of reduction in infarct volume were observed 1 wk after ischemia for single- and 5-day repeated-administrations of YM872. Because of the drug's short plasma half-life and high water solubility, YM872 disappeared from plasma quickly after the end of infusion and did not precipitate in the internal organs. This eliminates the potential problem of a drug depot that might provide sustained release of the drug as seen with NBQX (Xue *et al.*, 1994; Nurse and Corbett, 1996). These results therefore suggest that it is sufficient to maintain adequate plasma levels of YM872 for not more than

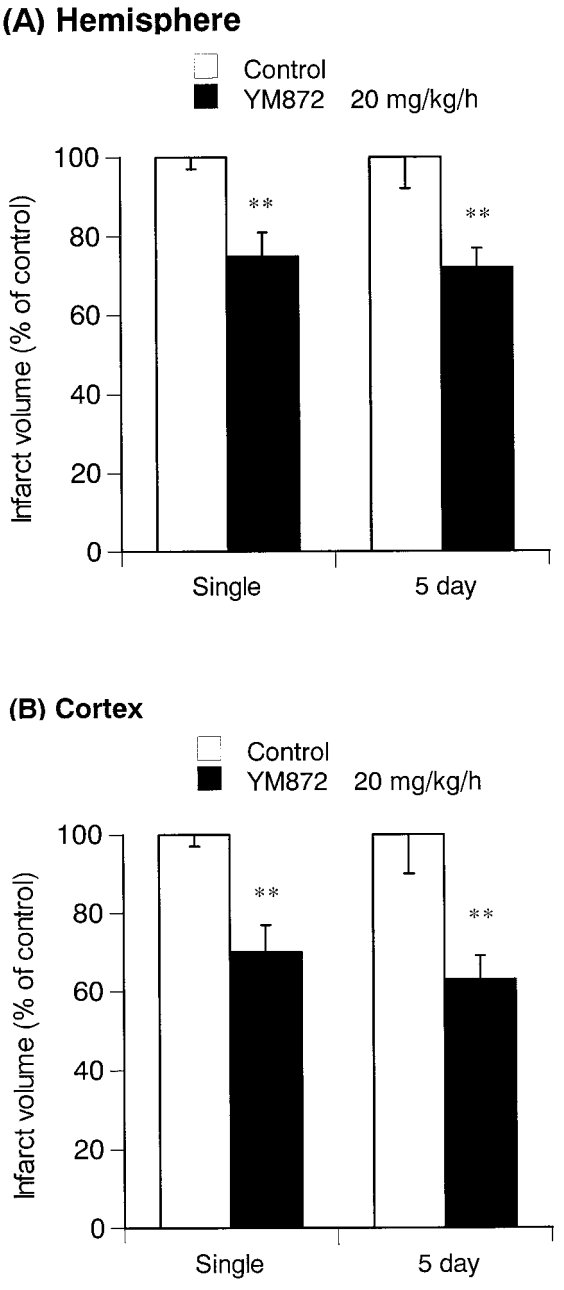

Fig. 5. Neuroprotective effect of YM872 on the volume of ischemic damage in (A) the cerebral hemisphere and (B) the cerebral cortex one week after MCA occlusion in rats. In single-administration groups (n = 8 in each group), saline or YM872 (20 mg/kg/hr) was i.v. infused for 4 hr starting 5 min after the onset of ischemia. In 5-day administration groups (n = 8 in each group), saline or YM872 (20 mg/kg/hr) was i.v. infused for 4 hr starting 5 min after the onset of ischemia and was then infused for 4 hr once daily over the after 4 days. ** $P < .01$ *vs.* respective controls. The neuroprotective efficacy of the 5-day administration of YM872 was almost same as that observed with the single-administration group.

4 hr after the onset of ischemia to obtain maximal therapeutic benefit in this experimental model. In preliminary experiments (Kayama *et al.*, unpublished data), administration of YM872 at a rate of 20 mg/kg/hr for either 4 or 24 hr starting 5 min after occlusion of MCA produced a brain concentration of approximately 300 ng/g (≈0.9 μM), at which the drug *in vitro* selectively blocks AMPA receptors (K_i = 0.096 μM) without blocking NMDA receptors ($K_i > 100$ μM) (Kohara *et al.*, 1996). This indicates that the neuroprotective effect of

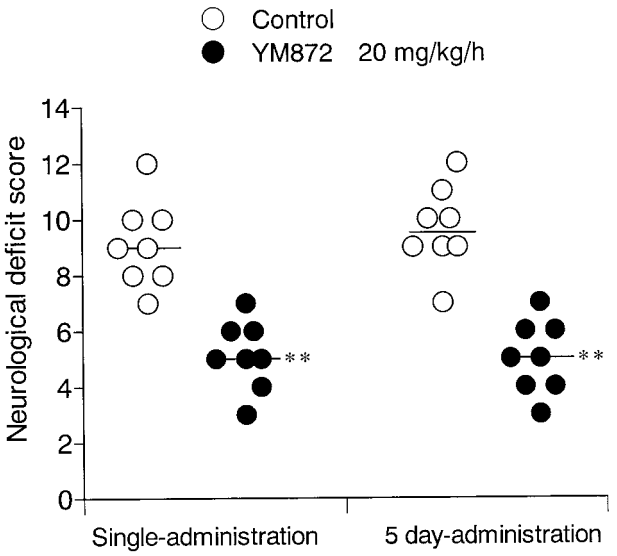

Fig. 6. Effect of YM872 on the neurological deficit score 1 wk after MCA occlusion in rats. In single-administration groups ($n = 8$ in each group), saline or YM872 (20 mg/kg/hr) was once i.v. infused for 4 hr starting 5 min after the onset of ischemia. In 5-day administration groups ($n = 8$ in each group), saline or YM872 (20 mg/kg/hr) was i.v. infused for 4 hr starting 5 min after the onset of ischemia and was then infused for 4 hr once daily over the following 4 days. ** $P < .01$ *vs.* respective controls. Similar extent of reduction was observed between single- and 5-day repetitive administrations of YM872.

YM872 in this study was in accord with its selective AMPA receptor blockade potency. These data also suggest that the AMPA receptor may play a key role in the early phase of ischemia and that neuroprotection by YM872 should provide cover during this critical period in rats.

Investigation of the therapeutic time window for YM872 showed that i.v. infusion at a rate of 20 mg/kg/hr for 4 hr gave significant protection even when initiated 2 hr after ischemia. A slight reduction in infarct volume was still seen when YM872 administration was delayed by 4 hr, although this was not statistically significant. In a further experiment, YM872 was infused over 20 hr starting 4 hr after ischemia. A slight reduction in infarct volume was again recognized, but still remained nonsignificant. Therefore, the maximum time that therapy could be delayed and still produce the desired result was 2 hr. NBQX was reported to be effective when the drug was given by multiple i.p. or i.v. administrations starting 90 min after the onset of ischemia in rats with transient or permanent MCA occlusions (Xue *et al.*, 1994; Graham *et al.*, 1996). In the study by Xue *et al.* (1994), a 4-hr i.v. infusion of NBQX was found to be effective starting 1 hr after ischemia. However, in these studies, the maximum time for initiation of drug administration had not been assessed. Taking account of the notion that the time window in humans after onset of symptoms could be substantially longer than that in rats (Pulsinelli, 1992), our results suggest that, if patients receive appropriate pharmacological intervention after ischemic onset, it would be possible that the extent of ischemic injury can be attenuated.

It is well known that the elevation of $[Ca^{++}]_i$ after increased glutamate in synaptic clefts plays a crucial role in ischemia-induced neuronal damage (Siesjö, 1981). AMPA antagonists are thought to demonstrate their neuroprotective effects by reducing this elevation of $[Ca^{++}]_i$ by blocking several pathways mediating its influx (Gill, 1994; Kohara *et al.*, 1996). These pathways include: 1) the NMDA receptor, which is voltage-dependently modulated by Mg^{++} under resting conditions (Wong and Kemp, 1991); 2) VSCCs on postsynaptic membranes (Miller, 1991); 3) reversal of $2Na^+/Ca^{++}$ exchanger (Siesjö and Bengtsson, 1989) and 4) AMPA receptors lacking the GluR2 subunit, which is also permeable to Ca^{++} (Hollmann *et al.*, 1991; Verdoorn *et al.*, 1991). Time course measurements of calcium homeostasis in rats using calcium-calmodulin binding after permanent MCA occlusion showed that an ischemia-induced $[Ca^{++}]_i$ accumulation was noted by 1 hr, reached a maximum by 4 hr, and remained at this level up to 24 hr after the ischemic onset (DeGraba *et al.*, 1993). Our results, therefore, suggest that the elevation of $[Ca^{++}]_i$ within 4 hr after focal cerebral ischemia is the most important factor in the progression of neuronal damage and that the blockade of AMPA receptors by YM872 for 4 hr could keep $[Ca^{++}]_i$ below toxic levels in surviving cells of the ischemic penumbra. The results of DeGraba *et al.* (1993) showing a window of opportunity for therapeutic intervention within 4 hr after ischemic onset also support the observation that the time window of efficacy for YM872 was up to 2 hr after the onset of ischemia.

It has been reported that hypothermia during or after ischemia may ameliorate ischemic injury (Xue *et al.*, 1992). It is also known that the change in brain temperature in awakening animals subjected to focal cerebral ischemia parallels the rectal temperature (Xue *et al.*, 1992). Therefore, in our experiments, rectal temperature was maintained within the normothermic range by means of a heating lamp during MCA occlusion surgery and infusion periods. In addition, some rats treated with YM872 at a rate of 20 mg/kg/hr, at which its neuroprotective effect was observed, had both brain and rectal temperatures measured during a 24-hr infusion period. There were no significant differences in these temperatures between the saline- and YM872-treated rats. Therefore, the neuroprotective effect of YM872 is unlikely to be due to hypothermia.

In conclusion, this study demonstrated that YM872, a selective and potent AMPA antagonist with improved water solubility, affords very effective neuroprotection with a substantially short treatment duration in rats with focal cerebral ischemia. It was particularly notable that neuroprotection was maintained even when administration began as late as 2 hr after ischemic onset. Moreover, significant decreases in neurological deficits were observed 1 wk after ischemia. These data suggest that the AMPA receptor plays a crucial role in the early phase of ischemia and that histological neuroprotection afforded by its antagonist in this phase is associated with functional improvement as well. In these dosing regimens, the administration of YM872 did not induce such adverse effects as physiological changes, serious behavioral abnormalities or nephrotoxicity. Taken together, our results indicate additional clinical promise for AMPA antagonists in treating acute ischemic stroke.

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