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Effect of SA4503, a novel σ_1 receptor agonist, against glutamate neurotoxicity in cultured rat retinal neurons

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Abstract

We examined the effects of σ_1 receptor agonists against glutamate-induced neurotoxicity in cultured retinal neurons. Primary cultures obtained from fetal rat retinas (16–19 d gestation) were used. The neurotoxic effect of glutamate was quantitatively assessed using the trypan blue exclusion method. A brief exposure of retinal cultures to glutamate (500 μM) led to delayed neuronal cell death. The glutamate-induced neurotoxicity was inhibited by (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo-[*a*,*b*]-cyclohepten-5,10-imine hydrogen maleate (MK-801). The σ_1 receptor agonists, 1-(3,4-dimethoxyphenethyl)-4-(3-phenylpropyl)-piperazine dihydrochloride (SA4503) and (+)-pentazocine at a concentration range of 0.1 ~ 100 μM reduced the glutamate-induced neurotoxicity in a dose-dependent manner. In addition, the neuroprotective effects of both SA4503 and (+)-pentazocine were antagonized by co-treatment with *N*,*N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethylamine monohydrochloride (NE-100), a putative σ_1 receptor antagonist. These findings suggest that σ_1 receptor agonists protect retinal cells against glutamate-induced neurotoxicity. © 1998 Elsevier Science B.V.

Keywords: Glutamate; SA4503; (+)-Pentazocine; Retinal cell culture; σ_1 Receptor subtype

1. Introduction

Martin et al. (1976) proposed the existence of the σ receptor as a novel opioid receptor subtype responsible for the psychotomimetic effects of benzomorphans such as (±)-pentazocine, cyclazocine and *N*-(±)-allylnormetazocine (SKF-10,047). The σ receptor is now established as a unique site, because it was shown that the characteristic behavioral changes caused by these benzomorphans were not antagonized by the classic opioid receptor antagonist, naloxone. Moreover, these drugs were found to bind to a unique non-opioid binding site, distinct from the phencyclidine receptor on the *N*-methyl-D-aspartate (NMDA) receptor channel complex (Walker et al., 1990). Recent studies indicated that the σ receptor had two distinct sites, termed σ_1 and σ_2 (Quirion et al., 1992; McCann et al., 1994).

Many studies have been carried out to elucidate the functional role of σ receptor subtypes. It has been suggested that, in the brain, σ receptor ligands exert a neuroprotective effect against ischemic injury (Rao et al., 1990; Pontecorvo et al., 1991). For example, (+)-SKF-10,047, a σ_1 receptor agonist (Itzhak, 1994), was reported to protect from the neurotoxicity induced by cerebral ischemia in mongolian gerbils and that induced by exposure to glutamate, one of the major neurotransmitters of ischemic neurotoxicity, in cultured rat cerebellar neurons (Lysko and Feuerstein, 1990; Lysko et al., 1992a,b). It was also reported that several σ receptor ligands protected from the neuronal cell death elicited by glutamate, hypoxia and/or NMDA, an agonist for the NMDA receptor channel complex, which is a glutamate receptor subtype involved in ischemic neurotoxicity, in cultured rat cortical neurons (DeCoster et al., 1995; Lockhart et al., 1995). Moreover, it was shown that the neuroprotective effects induced by σ receptor ligands against glutamate-induced neurotoxicity involved the σ_1 receptor subtype, because there was a correlation between the neuroprotective potency of σ receptor ligands and their binding affinity for the σ_1 receptor subtype (DeCoster et al., 1995). These findings indicate that the σ_1 receptor subtype is involved in the neuroprotective effect against ischemic injury in the

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brain. Therefore, we have synthesized the novel σ_1 receptor ligand, 1-(3,4-dimethoxyphenethyl)-4-(3-phenylpropyl)-piperazine dihydrochloride (SA4503) (Matsuno et al., 1996; Senda et al., 1996). We have also shown that SA4503 acts as an agonist for the σ_1 receptor subtype, because the inhibitory potency of SA4503 for [^{3}H](+)-pentazocine binding was weakened in the presence of guanosine 5′-*o*-(3-thiotriphospate) (GTPγS) (Matsuno et al., 1996); this action of SA4503 is similar to that of (+)-pentazocine, a prototypic σ_1 receptor agonist (Monnet et al., 1992; Cagnotto et al., 1994).

Several studies concerning the σ receptor in the eye have been reported. Particularly, it was suggested that the σ receptor exists in the retina, which is an integral part of the central nervous system. For example, it was reported that specific binding of both [^{3}H]1,3-di-(2-tolyl)guanidine ([^{3}H]DTG) and [^{3}H](+)-3(3-hydroxyphenyl)-*N*-(1-propyl)piperidine ([^{3}H](+)-3-PPP) was recognized in the rat retinal membrane (Pingping et al., 1992). Because DTG is a ligand with high affinity and selectivity for both σ_1 and σ_2 receptor subtypes (Quirion et al., 1992), and (+)-3-PPP is a selective σ_1 receptor ligand (Quirion et al., 1992), respectively, this report suggested that the σ_1 receptor subtype existed in the retina. We also showed that a high density of both σ_1 and/or σ_2 receptor subtypes was present in the bovine retinal membrane (Senda et al., 1997a). Thus, while σ receptor subtypes exist in the retina, their functional roles have never been examined. In the present study, therefore, to elucidate whether the σ_1 receptor subtype is involved in retinal neuroprotection, we investigated the effects of the σ_1 receptor agonists, SA4503 and (+)-pentazocine, against glutamate-induced neurotoxicity in rat cultured retinal neurons.

2. Materials and methods

The procedures involving animals and their care were conducted in conformity with the institutional guidelines which comply with the Guide for the Care and Use of Laboratory Animals (NIH Publication, No. 85-23, 1985)

2.1. Cell cultures

Primary cultures obtained from the retinas of fetal rats (16–19 d gestation) were used (Kashii et al., 1994; Kikuchi et al., 1995). The retinal tissue was minced, mechanically dissociated, and filtered through a stainless steel mesh to obtain single-cell suspensions (about 7.0×10^6 cell/ml). One hundred μl of the cell suspension was plated on plastic coverslips with polyethyleneimine coating. Cultures were incubated in Eagle's minimal essential medium (Eagle's salts; Nissui, Tokyo) supplemented with 10% heat-inactivated fetal calf serum (1–7 d after plating), or 10% heat-inactivated horse serum (8–11 d after plating) containing 2 mM L-glutamine, 11 mM glucose, 24 mM sodium bicarbonate, and 10 mM 4-(2-hydroxy-ethyl)-1-piperazine-ethane-sulpholic acid (HEPES). The retinal cultures were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide–95% air. After 5 d culture, the growth of non-neuronal cells was terminated by the addition of 10 μM cytosine arabinoside. In this study, only cultures maintained for 10–11 d in vitro and only isolated cells were used. Clusters of cells were excluded from the results because such cells could not be used for histologic experiments. Most of the isolated cells were shaped like neurons. Previous study had also revealed that isolated retinal cells cultures maintained under these conditions consisted mainly of neuronal cells, and that these neuronal cells included about 55% of amacrine cells, and no ganglion cells (Kashii et al., 1994). Moreover, these cells respond to both NMDA and non-NMDA receptor agonists (Ujihara et al., 1993). Therefore, such neurons correspond to transient amacrine cells in the tiger salamander retina (Dixon and Copenhagen, 1992).

2.2. Measurement of neurotoxicity

The neurotoxic effect of glutamate was quantitatively assessed by the trypan blue exclusion method as described earlier (Kashii et al., 1994; Kikuchi et al., 1995). Experiments were performed in the above medium without serum (standard medium) at 37°C. Glutamate (500 μM), a neurotoxic agent, was applied to cultures for 10 min. Then, the cultures were incubated in the standard medium for 1 h. We used SA4503 and (+)-pentazocine as selective σ_1 receptor agonists, *N*,*N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethylamine monohydrochloride (NE-100) (Okuyama et al., 1993; Chaki et al., 1994) as a selective σ_1 receptor antagonist, and (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo-[*a*,*b*]-cyclohepten-5,10-imine hydrogen maleate (MK-801) (Wong et al., 1986) as a non-competitive antagonist for the NMDA receptor channel complex, respectively. They were applied to the cultures for 10 min prior to the exposure to glutamate and were also applied for 10 min during the exposure to glutamate. After completion of the treatment, the cell cultures were stained with 1.5% Trypan blue solution at room temperature for 10 min and then fixed with isotonic formalin (pH 7.0, 2–4°C). The fixed cultures were rinsed with physiological saline and examined under Hoffman modulation microscopy. More than 200 cells were randomly counted to determine the viability of the cell cultures. The viability of the cultures was given by the number of unstained cells (viable cells) as a percentage of the total number of cells counted (viable cells plus nonviable cells).

2.3. Statistical analysis

The results are expressed as the mean values ± S.E.M. for cell viability from five coverslips. Statistical comparisons were made with one-way analysis of variance, fol-

lowed by Tukey's test for multiple comparisons. Differences in P values less than 0.05 were considered to be statistically significant.

2.4. Drugs

The following drugs were used: SA4503, (+)-pentazocine and NE-100 (synthesized in our laboratory); L-glutamic acid monosodium salt (glutamate) (Nacalai Tesque, Kyoto); MK-801 (Research Biochemicals Int., Wayland, MA). Other chemicals and reagents of analytical grade were obtained from commercial suppliers.

We prepared stock solutions of the drugs as follows: glutamate, SA4503, NE-100 and MK-801 were dissolved in distilled water. (+)-Pentazocine was dissolved in 1 M HCl and neutralized with 1 M NaOH. Stock solutions of each drug were then diluted 100-fold into standard medium to the final concentrations indicated in Section 3.

3. Results

3.1. Effects of SA4503, (+)-pentazocine and MK-801 on glutamate-induced neurotoxicity

Fig. 1 shows an example of the effects of SA4503 and (+)-pentazocine on glutamate-induced neurotoxicity. The cells were exposed briefly (10 min) to glutamate (500 μM) and were incubated for 1 h in a standard medium. Most cells in the cultures examined were viable (Fig. 1A); however, exposure to glutamate markedly reduced cell viability (Fig. 1B). In a pilot study, maximum protection

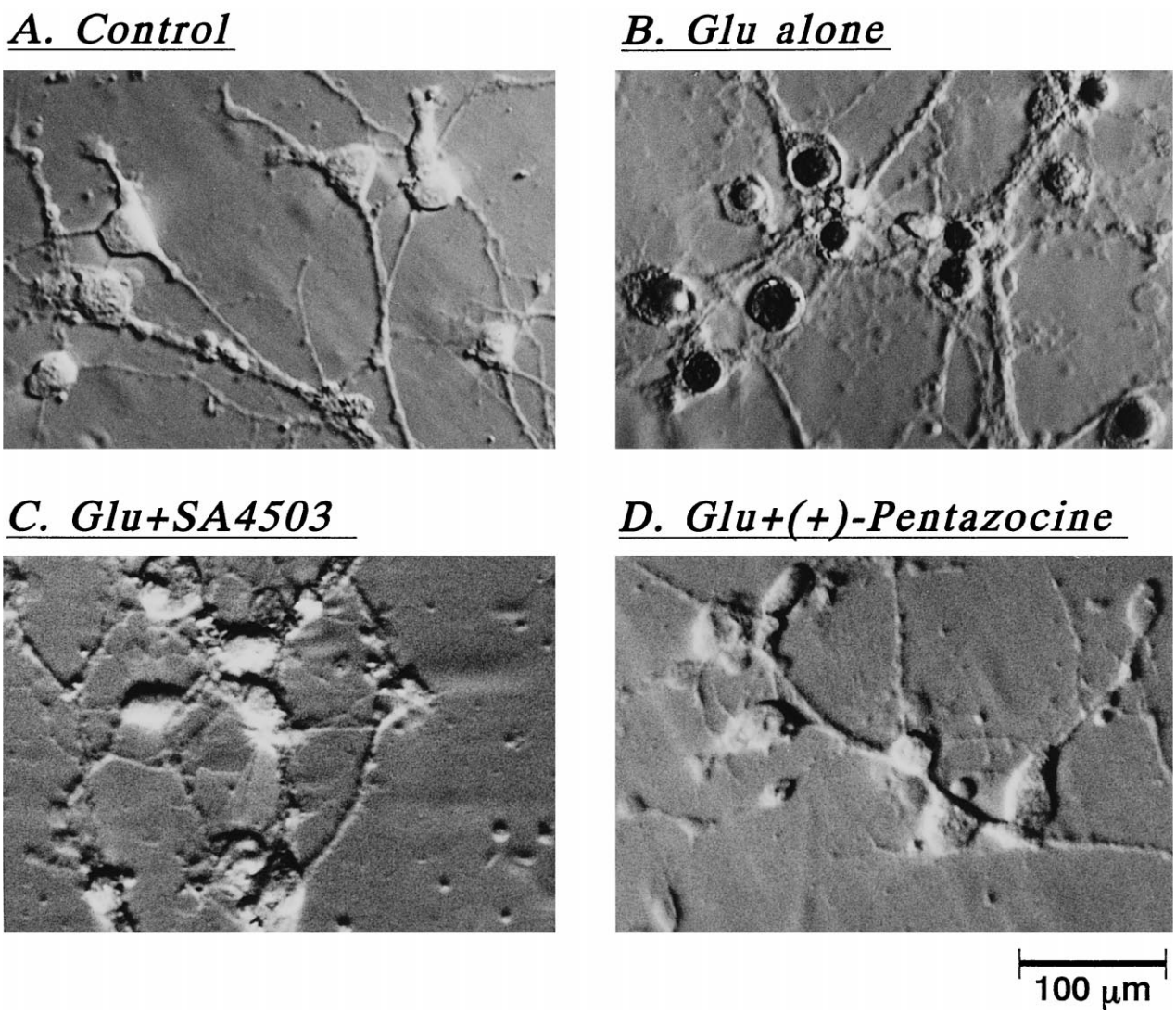

Fig. 1. Photomicrographs showing the effects of SA4503 and (+)-pentazocine on glutamate (Glu)-induced neurotoxicity. All cultures were photographed after trypan blue staining followed by formalin fixation using Hoffman modulation microscopy. (A) Control (non-treated) cells. Cells were stained without the application of Glu. (B) Cells treated with Glu (500 μM), followed by a 1 h incubation with Glu-free medium (standard medium). (C, D) Cells treated with Glu plus SA4503 (100 μM) (C) or (+)-pentazocine (100 μM) (D), followed by 1 h incubation with standard medium. Calibration bar = 100 μm.

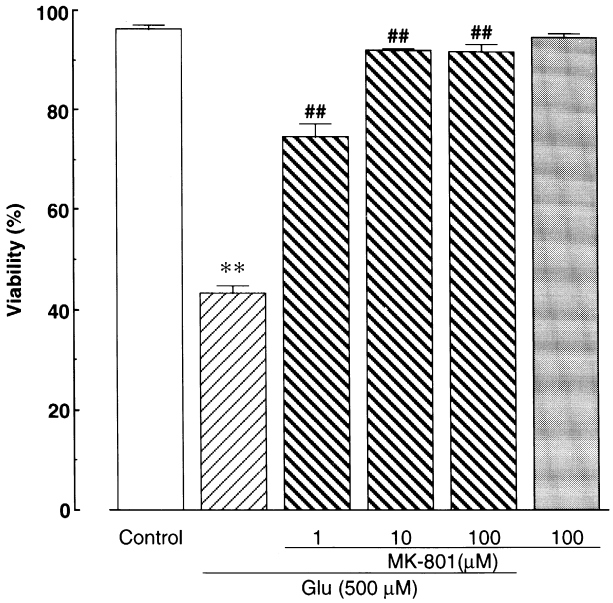

Fig. 2. The protective effect of MK-801 against glutamate (Glu)-induced neurotoxicity in rat cultured retinal neurons. Cells were treated with Glu (500 μM) for 10 min, followed by a 1 h incubation with standard medium. MK-801 was applied to the cultures for 10 min prior to Glu and was also applied for 10 min during the exposure to Glu. Cell viability was assessed by Trypan blue exclusion. Values are expressed as means ± S.E.M. of the cell viability from five coverslips. $^{**}P < 0.01$ as compared with the control group. $^{\#\#}P < 0.01$ as compared with the Glu alone group.

by SA4503 was observed when the drug was applied to the cultures for 10 min prior to the exposure to glutamate and also applied for 10 min during the exposure to glutamate. The treatments with 100 μM of SA4503 and (+)-pentazocine both markedly reduced the cell death induced by glutamate (Fig. 1C and D).

Figs. 2 and 3 summarize the protective effects of MK-801, SA4503 and (+)-pentazocine against glutamate-induced neurotoxicity. Cell viability was significantly reduced by the exposure to glutamate. This glutamate-induced neurotoxicity was significantly inhibited by the treatment with MK-801, a non-competitive antagonist for the NMDA receptor channel complex (Fig. 2). SA4503 and (+)-pentazocine, in concentrations ranging from 0.01 to 100 μM, dose dependently reduced the glutamate-induced neurotoxicity (Fig. 3). Significant protective effects of both SA4503 and (+)-pentazocine against glutamate-induced neurotoxicity were observed at concentrations of more than 0.1 μM. None of these drugs alone at 100 μM (no glutamate exposure) had any effect on cell viability (Figs. 2 and 3), indicating that drug concentrations of 100 μM or less did not exhibit intrinsic neurotoxicity.

3.2. Antagonism with NE-100 against the protective effects of SA4503 and (+)-pentazocine from glutamate-induced neurotoxicity

To determine whether the SA4503- and (+)-pentazocine-induced neuroprotective effects were mediated through the σ_1 receptor, the antagonistic effect of NE-100 was studied. Cell viability was not affected by NE-100 alone at a concentration of 100 μM (Fig. 4A). The protective effects of SA4503 (100 μM) against glutamate-in-

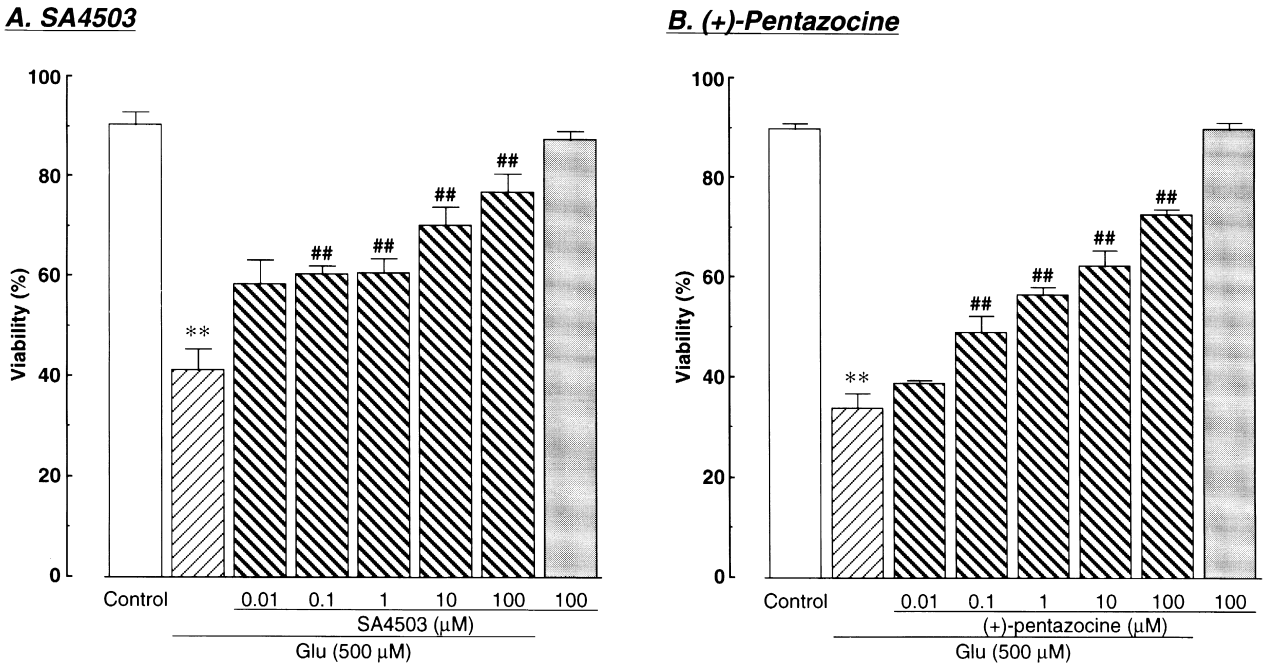

Fig. 3. The protective effects of SA4503 (A) and (+)-pentazocine (B) against glutamate (Glu)-induced neurotoxicity in rat cultured retinal neurons. SA4503 (0.01 ~ 100 μM) or (+)-pentazocine (0.01 ~ 100 μM) was applied to the cultures for 10 min prior to Glu and was also applied for 10 min during the exposure to Glu. Values are expressed as means ± S.E.M. of the cell viability from five coverslips. For further details, see the legend to Fig. 2. $^{**}P < 0.01$ as compared with the control group. $^{\#\#}P < 0.01$ as compared with the Glu alone group.

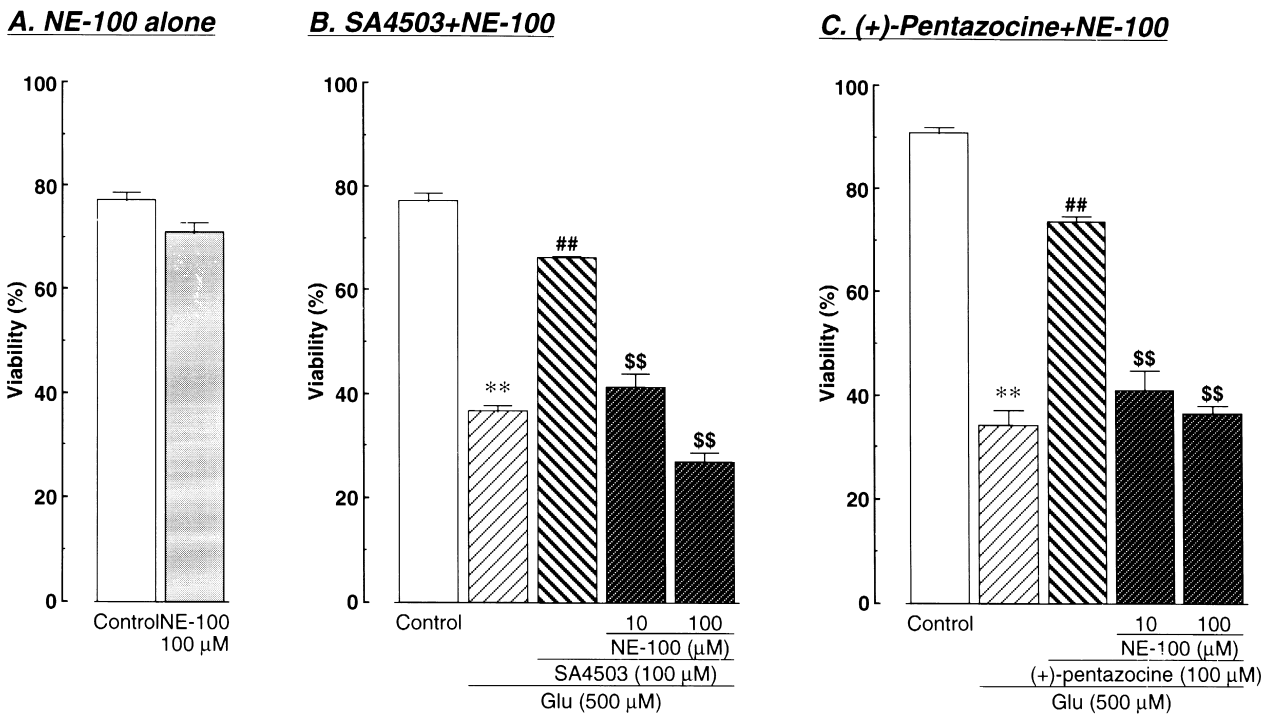

Fig. 4. Antagonism by NE-100 of the protective effects of SA4503 and (+)-pentazocine against glutamate (Glu)-induced neurotoxicity in rat cultured retinal neurons. SA4503 (100 μM), (+)-pentazocine (100 μM) and NE-100 (10 and 100 μM) were applied to the cultures for 10 min prior to Glu and were also applied for 10 min during the exposure to Glu. Values are expressed as means ± S.E.M. of the cell viability from five coverslips. For further details, see the legend to Fig. 2. $^{**}P < 0.01$ as compared with the control group. $^{\#\#}P < 0.01$ as compared with the Glu alone group. $^{\$\$}P < 0.01$ as compared with the Glu + SA4503 or (+)-pentazocine group.

duced neurotoxicity were dose dependently reduced by co-treatment with NE-100 (10 and 100 μM) (Fig. 4B). The effect of (+)-pentazocine was also antagonized by NE-100 (Fig. 4C).

4. Discussion

The present results showed that SA4503 and (+)-pentazocine reduced the neurotoxicity induced by glutamate in cultured retinal neurons, and the neuroprotective effects of both drugs were antagonized by NE-100. It has been reported that SA4503 (Matsuno et al., 1996; Senda et al., 1996, 1997a), (+)-pentazocine (De Costa et al., 1989; Walker et al., 1990; Bowen et al., 1993; Matsuno et al., 1996; Senda et al., 1996, 1997a) and NE-100 (Okuyama et al., 1993; Chaki et al., 1994; Tanaka et al., 1995) had high affinity for the σ_1 receptor subtype and bound to no other receptors (including the NMDA receptor channel complex) with high affinity. Many other reports have indicated that SA4503 and (+)-pentazocine act as selective agonists for the σ_1 receptor subtype (Steinfels et al., 1988, 1989; Itzhak, 1989; Monnet et al., 1992; Gonzalez-Alvear and Werling, 1994, 1995; Matsuno et al., 1996). In contrast, Okuyama et al. (1993) showed that NE-100 antagonized the head-weaving behavior induced in mice by (+)-SKF-10,047 which was reported to show an agonistic property for the σ_1 receptor subtype (Itzhak, 1994). We had also shown that NE-100 antagonized the SA4503-, (+)-pentazocine- and (+)-SKF-10,047-induced ameliorating effects against scopolamine-induced memory impairment in rats and mice (Senda et al., 1996, 1997b). In addition, it was reported that NE-100 had no effect on the several actions mediated through the NMDA receptor channel complex (Maurice et al., 1994; Debonnel and De Montigny, 1996; Monnet et al., 1996; Okuyama et al., 1996). Thus, we used SA4503 and (+)-pentazocine as selective agonists for the σ_1 receptor subtype and NE-100 as a selective antagonist for the σ_1 receptor subtype. Therefore, we propose that the agonistic activation of the σ_1 receptor subtype produces neuroprotection against glutamate-induced neurotoxicity in the retina.

Since there is no information concerning the retinal σ_1 receptor subtype, except for the findings from binding studies, the exact mechanism(s) by which σ_1 receptor subtypes act to mediate their neuroprotective function in the retina remains unclear. However, it was suggested that, in the brain, the σ_1 receptor subtype-mediated neuroprotection might be the result of modulatory effects on actions induced by the NMDA receptor channel complex. For example, it was reported that several σ receptor ligands, including σ_1 receptor agonists such as (+)-pentazocine

and (+)-SKF-10,047, attenuate the NMDA-evoked inward currents in mouse hippocampal neurons (Fletcher et al., 1995) and reduce the NMDA-induced neurotoxicity in cultured neurons (Lockhart et al., 1995). In addition, Lysko et al. (1992b) showed that (+)-SKF-10,047 diminishes the intracellular calcium levels of NMDA-stimulated rat cultured cerebellar neurons in a dose-dependent manner. Previous studies (Kashii et al., 1994; Kikuchi et al., 1995) and the present findings also indicated that glutamate-induced neurotoxicity was mediated mainly through the NMDA receptor channel complex, because MK-801 completely antagonized glutamate-induced neurotoxicity. The excessive calcium influx induced by toxic activation of the NMDA receptor channel complex is well known to play a role in glutamate-induced neurotoxicity in the central nervous system (Ogura et al., 1988; Siesjö, 1988; Manev et al., 1989; Abele et al., 1990). Therefore, it is assumed that the protective effects of σ_1 receptor agonists against the glutamate-induced neurotoxicity in the retina result from modulating interactions with the NMDA receptor channel complex, probably through reduction of the NMDA-induced calcium influx.

As mentioned in Section 1, it is well known that synaptically released excitatory amino acid neurotransmitters, most likely glutamate, play a key role in ischemic and hypoxic brain damage and cause neuronal cell death (Abele et al., 1990; Choi and Rothman, 1990; Meldrum and Garthwaite, 1990). In fact, it was reported that cerebral ischemia caused a massive release of endogenous glutamate into the ischemic brain tissue (Benveniste et al., 1984), and that glutamate receptor antagonists, particularly NMDA receptor channel complex antagonists, markedly reduce brain injuries associated with ischemia (Simon et al., 1984; Meldrum, 1985; Foster et al., 1988; Gotti et al., 1990). It was also reported that, in the retina, glutamate was present as one of the retinal neurotransmitters in experimental animals (Neal, 1976; Brandon and Lam, 1983; Bloomfield and Dowling, 1985; Sarantis et al., 1988) and humans (Brandon and Lam, 1983; Davanger et al., 1991), that retinal ischemia increased the synaptically released glutamate (Johnson, 1974; Louzada-Junior et al., 1992; Neal et al., 1994), and that NMDA receptor channel complex antagonists protect from the neuronal damage elicited by retinal ischemia (Yoon and Marmor, 1989; Ei-Asrar et al., 1992), as they do in the brain. These findings indicate that the damage to retinal neurons induced by ischemia and/or hypoxia involves an excess release of glutamate. In the present study, it was shown that agonist activation of the σ_1 receptor subtype was involved in the neuroprotection against glutamate-induced neurotoxicity. Therefore, the possibility can be considered that σ_1 receptor agonists may be useful as therapeutic drugs against retinal diseases with neuronal cell death due to ischemia, such as central and branch retinal artery occlusion, diabetes mellitus, age-related macular degeneration, hemoglobinopathies, and various types of glaucoma.

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