Activation of the GABA_A Receptor Ameliorates the Cochlear Excitotoxicity Caused by Kainic Acid in the Guinea Pig

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Excitotoxicity is a major neurotoxic mechanism in cochlear disorders, including cochlear ischemic injury and acoustic injury. Kainic acid (KA), an excitatory amino acid, can damage glutamatergic neurons. The application of KA to the round window membrane, an opening of the cochlear bony labyrinth to the middle ear, induces excitotoxicity of cochlear afferent dendrites and significantly decreases the amplitude of compound action potential of the cochlea (CAP), a cochlear potential generated by activation of the auditory nerve fibers. On the other hand, muscimol, a gamma-aminobutyric acid (GABA)_A receptor agonist, is neuroprotective in excitotoxicity in the central nervous system. Here we studied whether activation of GABA receptor decreased the excitotoxicity of the cochlea caused by KA. KA (10 mM) was applied to the round window membrane of guinea pigs for 30 minutes. Muscimol, bicuculline, a GABA_A receptor antagonist, or baclofen, a GABA_B receptor agonist, was given at the onset of KA application. The threshold shift of CAP was examined with chronically implanted electrodes. Application of KA significantly elevated the CAP threshold on day 1 and day 3 as compared with the pre-application level. Muscimol significantly decreased the KA-induced CAP threshold shifts. Furthermore, this protective effect of muscimol was inhibited by the application of bicuculline, a GABA_A receptor antagonist. However, baclofen, a GABA_B receptor agonist, did not affect the CAP threshold shifts caused by KA. These results suggest that activation of GABA_A receptor could prevent cochlear excitotoxicity. GABA_A receptor activation may represent an effective treatment of cochlear ischemic injury and acoustic injury. ——— Kainic acid; Excitotoxicity; Muscimol; Gamma-aminobutyric acid; Cochlea.

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Since the late 1960s researchers have observed that glutamate and glutamate receptor agonists can cause neuronal death in the brain. Olney et al. (1969, 1970) found that glutamate toxicity produced marked dendrite swelling, and the pathological mechanism was termed ‘excito-

toxicity’ whereby excessive accumulation of excitatory amino acids produced neurodegeneration. Excitotoxicity is now considered to be a predominant mechanism of neuronal death in diseases of the central nervous system (CNS), such as stroke, CNS trauma, epilepsy and Parkinson’s

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disease.

There is a large body of evidence indicating that glutamate is the afferent neurotransmitter responsible for fast synaptic transmission from the inner hair cells to afferent nerve endings in the cochlea. Expression of ionotropic glutamate receptors, including N-methyl-D-aspartate (NMDA), α -amino-3-methyl-4-isoxazole-propionic acid (AMPA) and kainite receptors, has been demonstrated in the spiral ganglion cells. Various studies support the idea that kainic acid (KA) can temporarily or permanently damage glutamatergic neurons. The application of KA to the round window membrane induces excitotoxicity of cochlear afferent dendrites and significantly decreases the compound action potential (CAP) (Zheng et al. 1999).

On the other hand, Gamma-aminobutyric acid (GABA) is known to be the major inhibitory neurotransmitter in the central nervous system. The GABA receptors are subdivided into two main categories, termed GABA_A and GABA_B, on the basis of their pharmacological characteristics. The existence of GABAergic synapses in the organ of Corti of the mammalian cochlea has been proved by the immunoreactivity of either the neurotransmitter GABA or of glutamic acid decarboxylase, a synthesizing enzyme of GABA (Fex et al. 1984; Eybalin et al. 1989). Recent studies have demonstrated that the GABA_A receptor was present in afferent nerve terminals under inner hair cells (Yamamoto et al. 2002). Since muscimol, a GABA_A receptor agonist, reduces the activation of the cochlear afferent nerves induced by AMPA and NMDA (Arnold et al. 1998), GABA is considered to regulate afferent nerve transmission. These findings suggest the potential protective effects of GABA receptor agonists on the excitotoxicity of the cochlea.

In the present study, we examined the protective effect of muscimol and baclofen, a GABA_B receptor agonist, on the cochlear excitotoxicity caused by KA.

MATERIALS AND METHODS

Animal preparation

Guinea pigs (250–300 g) with normal Preyer pinna

reflex were used in the present study. The animals are anesthetized with an intraperitoneal injection of 28 mg/kg of pentobarbital sodium (Nembutal®, Abott Laboratories, North Chicago, IL, USA) and with the local injection of lidocaine (Xylocaine®, Fujisawa Pharmaceutical co., Osaka, Japan) at the surgical site. The rectal temperature was kept at $37 \pm 1^\circ\text{C}$ using a heat pad. The care and use of the animals used in this study were approved by the Animal Experiment Committee at University of Tsukuba.

Implantation of electrodes and the measurement of compound action potential (CAP)

After postauricular incision was made, the facial nerve was identified and was extracted from the right facial canal. A silver electrode coated with Teflon was implanted within the facial canal in order to consecutively measure CAP (Wada et al. 1999). The test stimuli for CAP measurement were pure tones of 2, 4, 8, 12 or 16 kHz (rise and fall times: 1 millisecond, duration: 10 milliseconds) delivered in the open field system (Tabuchi et al. 2003). The sound pressure level needed to evoke a CAP of $4 \mu\text{V}$ in amplitude was defined as the threshold. The CAP threshold was measured immediately and one week after the implantation of electrodes. If the difference in the CAP threshold was less than 9 dB at all frequencies measured, chemical agents such as KA and GABA agonists were locally applied to the round window membrane. The CAP threshold was then examined on day 1, day 3 and day 7 after the drug application.

Histological evaluation of excitotoxicity

For histological studies, 25 animals were sacrificed 1 day after the application of drugs. In addition, 5 animals each treated with 10 mM KA alone were sacrificed at 3 days and 7 days after the application. After making a tiny hole at the cochlear apex, with 4% paraformaldehyde the cochleae were perfused from the round window to the apex. The cochleae were then removed and embedded in paraffin. Midmodiolar sections of $5 \mu\text{m}$ thickness were stained with hematoxylin and eosin.

Drug administration

KA, muscimol, bicuculline and baclofen were obtained from Sigma Japan (Tokyo, Japan). The composition of artificial perilymph used in this study was as follows; 137 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 1 mM NaHCO_3 , 11 mM glucose, pH 7.4 (Bobbin et al. 1974 and Tabuchi et al. 2002). For the application

of KA, a hole of approximately 3 mm in diameter was made in the auditory bulla, through which 30 μ l KA (concentration: 10 mM in artificial perilymph) was locally applied to the round window membrane. The KA was left on the round window membrane for 30 minutes (Zheng et al. 1999) and then washed out with artificial perilymph. After the application of KA, the auditory bulla was closed using polycarboxylate cement (Carbo cement®, Matsukaze Co., Kyoto, Japan). Muscimol (1 mg/kg), bicuculline (2 mg/kg) or baclofen (2 or 4 mg/kg) was given intraperitoneally to the animals at the onset of KA application.

Data analysis

The number of animals was 5 in an every experimental group. All results are presented as the mean $\pm$ SEM. The CAP thresholds were assessed with two-way ANOVA and the Scheffe test. *P* values less than 0.05 were considered significant.

RESULTS

Confirmation of the condition of implanted electrodes

The condition of the implanted electrodes and the effect of surgeries on the CAP thresholds were checked using 5 animals. When artificial perilymph (30 μ l) alone was applied to the round window membrane for 30 minutes, the CAP threshold did not significantly change during 1 week after the implantation of electrodes (two-way ANOVA: *P* > 0.05 among time points, data not shown).

Temporary CAP threshold shifts induced by KA

The time course of the CAP threshold before and after KA application was examined using 15 animals. The application of 1, 10 or 60 mM KA (*n* = 5 in each group) to the round window membrane significantly increased the CAP thresholds on day 1 (Fig. 1). The CAP threshold had recovered to the pre-application level by day 7 in all groups. Although the CAP threshold shift was larger in the 10 and 60 mM groups than the 1 mM group on day 1 and day 3, there was no statistically significant difference in the CAP threshold shift between the 10 and 60 mM groups at any time point examined (two-way ANOVA: *p* < 0.05, Scheffe test: *p* < 0.05 at 1 mM).

Fig. 1. Time course of CAP thresholds after KA application. Application of KA (10 mM and 60 mM, *n* = 5 in each group) to the round window membrane increased CAP thresholds 1 and 3 days after the application. CAP thresholds recovered to the pre-application levels 7 days after the application. There was no statistically significant difference in the CAP threshold between the 10 and 60 mM groups at any time point examined (Scheffe test).

Effect of muscimol and bicuculline on CAP threshold shifts induced by KA

The effect of muscimol, a GABA_A receptor agonist, on the KA-induced CAP threshold was examined. The intraperitoneal administration of 1 mg/kg muscimol itself did not significantly affect the CAP thresholds (two-way ANOVA: $p > 0.05$, data not shown). As shown in Fig. 2, the CAP threshold shifts induced by 10 mM KA were significantly decreased by muscimol as compared with day 1 and day 3 ($n = 5$, two-way ANOVA and Scheffe test: $p < 0.01$ at day 1 and day 3).

The protective effect of muscimol was significantly inhibited by the application of the GABA_A receptor antagonist bicuculline on day 1 and day 3 ($n = 5$, 2 mg/kg, i.p., two-way ANOVA and Scheffe test: $p < 0.05$ between KA + muscimol and KA + muscimol + bicuculline). There was no statistically significant difference between the KA alone animals and the animals that were given KA, muscimol and bicuculline on days 1 through 7 (Scheffe test: $p > 0.05$).

Effect of baclofen on CAP threshold shifts induced by KA

The effect of baclofen, a GABA_B receptor agonist, on the KA-induced CAP threshold was examined. The intraperitoneal administration of 4 mg/kg baclofen *per se* did not significantly affect the CAP threshold ($n = 5$, data not shown). As shown in Fig. 3, there was no statistically significant difference in the CAP threshold shift between the groups treated with KA alone and KA followed by baclofen (2 or 4 mg/kg, $n = 5$ in each group) on day 1, 3 and 7.

Morphological effect of muscimol and baclofen on afferent dendrites in basal turn.

The effect of the GABA_A and GABA_B agonists on the morphology of afferent dendrites were examined. Normal pear-shaped inner hair cells were observed in the control cochlea (Fig. 4A). Swollen afferent nerve endings formed empty spaces beneath the inner hair cells in the KA-treated animals on day 1 (Fig. 4B). The swelling of afferent nerves decreased on day 3 (Fig. 4C) and almost diminished on day 7 (Fig.

Fig. 2. The effects of GABA_A agonist and antagonist on CAP threshold shifts induced by KA. CAP threshold shifts induced by 10 mM KA ($n = 5$) were decreased by GABA_A receptor agonist muscimol ($n = 5$, $**p < 0.01$). This protective effect of muscimol was inhibited by GABA_A receptor antagonist bicuculline ($n = 5$).

Fig. 3. The effect of GABA_B agonist baclofen on CAP threshold shifts induced by KA. GABA_B agonist baclofen (2 or 4 mg/kg, $n = 5$ in each group) did not ameliorate KA-induced excitotoxicity (two-way ANOVA).

4D). One mg/kg muscimol clearly inhibited this swelling of the afferent dendrite (Fig. 4E). The application of 2 mg/kg bicuculline in addition to muscimol antagonized the effect of muscimol (Fig. 4F). In contrast to the findings observed in the muscimol-treated animals, baclofen did not show any effect on the KA-induced swelling of afferent dendrites, and afferent dendrites were massively swollen beneath the inner hair cells in this group (Fig. 4G).

DISCUSSION

Zheng et al. (1999) reported that the application of KA to the round window membrane induced the excitotoxicity of afferent dendrites. They used 60 mM KA to induce excitotoxicity, and the results obtained in the present study were essentially the same as those of their study. Because there was no statistically significant difference in the time course of the CAP threshold shift between the 10 mM and 60 mM KA groups, we decided to examine the cochlear excitotoxicity at the concentration of 10 mM.

Muscimol significantly ameliorated the excitotoxicity of the cochlea induced by 10 mM KA. Muscimol decreased the KA-induced CAP threshold shift and the swelling of afferent nerve endings. This protective effect of muscimol was inhibited by the administration of bicuculline. It was concluded from these findings that the GABA_A receptor was involved in the protection of the cochlea from excitotoxicity. However, baclofen did not have any protective effect on the KA-induced cochlear excitotoxicity, suggesting that the GABA_B receptor did not have any effect on the cochlear excitotoxicity. It has been shown that the systemic administration of baclofen at the dosage of 1 to 2 mg/kg antagonizes glutamate excitotoxicity in the central nervous system (Slattery et al. 2005, Diaz et al. 2006 and Wojnicki et al. 2006). For this reason, we selected the administration dosage of baclofen in the present study.

In trigeminal motoneurons, the transmission of glutamatergic synapses was regulated by GABA by means of axo-dendritic synapses (Yang et al. 1997). In addition, Yamamoto et al. (2002)

Fig. 4. Swelling of afferent dendrites one day after KA application to the round membrane.
 A: Control cochlea. Normal pear-shaped inner hair cells were observed, but swelling of afferent nerve endings was not clear.
 B-D: Cochlea treated with KA. Massive swelling of the afferent dendrite (arrowhead) was observed in the animals treated with KA alone one day after the administration (B). The swelling was decreased by three days after the administration (C) and almost diminished after 7 days (D).
 E: Cochlea treated with KA and muscimol. Muscimol partially inhibited KA-induced swelling.
 F: Cochlea treated with KA, muscimol and bicuculline. Bicuculline antagonized the effect of muscimol, and afferent dendrites were massively swollen (arrowhead).
 G: Baclofen did not inhibit the swelling (arrowhead).

reported that GABA_A receptors were present in afferent nerve endings beneath inner hair cells. It may be assumed from those findings that GABA has the potential to regulate the activity of a glutamate receptor by means of axo-dendritic synapses. Further studies will be necessary to clarify the detailed mechanism of the inhibitory actions of GABA in the cochlea.

It has been demonstrated that the excitotoxicity of afferent dendrites plays important roles in cochlear ischemia (Pujol et al. 1990 and Tabuchi et al. 2002) and acoustic injury (Puel et al. 1998). Glutamate excitotoxicity often causes temporary threshold shifts in this type of injury. However, Sun et al. (2000) demonstrated that some cochlear excitotoxicity might be irreversible because the number of cochlear ganglion neurons decreased after exposure to a high concentration of KA. It is very important to establish pharmacological protective strategies about the excitotoxicity of the cochlea.

In conclusion, the present study suggests that the activation of GABA_A receptors, but not GABA_B receptors, can protect the cochlea from excitotoxicity.

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