L-Type Voltage-Gated Calcium Channel is Involved in the Pathogenesis of Acoustic Injury in the Cochlea

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Excessive calcium entry into cells leads to cell death, and voltage-gated calcium channels (VGCCs) are responsible for the calcium entry in the central nervous system. VGCC blockers inhibit excessive calcium entry and protect the central nervous system against various types of injury. The purpose of the present study was to identify these calcium channels that are responsible for acoustic injury of the cochlea. The effects of L- and T-type VGCC blockers on acoustic injury were examined. Female ddY mice, at 8 weeks of age, were used in this study. The animals were subjected to a 4-kHz pure tone of 128-dB sound pressure level (SPL) for 4 hours through an open field system inside a sound-exposure box. A L-type or T-type VGCC blocker was administered immediately before acoustic overexposure. The hearing ability was evaluated using the auditory brainstem response (ABR). ABR is an electrical signal evoked from the brainstem by the sound. After the final ABR measurement at two weeks after acoustic overexposure, cell nuclei in the organ of Corti were stained with propidium iodide, and hair cell loss was calculated in a region 3.66 mm from the apex. Each of four L-type VGCC blockers tested, i.e. diltiazem, verapamil, nicardipine and nimodipine, significantly improved shifts of the ABR threshold from the pre-exposure levels. In addition, each L-type VGCC blocker consistently decreased hair cell loss, but not a given T-type calcium blocker. The present findings suggest that the L-type VGCC is involved in the pathogenesis of acoustic injury in the cochlea. ——— voltage-gated calcium channel (VGCC) blockers; acoustic injury; L-type calcium channel; T-type calcium channel; auditory brainstem response (ABR).

Tohoku J. Exp. Med., 2009, 218 (1), 41-47. © 2009 Tohoku University Medical Press

Homeostatic control of Ca²⁺ is essential for cell survival. Like other organs, cells in the inner ear are thought to use both intra- and extracellular sources of calcium. At the plasma membrane of hair cells, transduction channels, ligand-gated channels, and voltage-gated calcium channels (VGCCs) facilitate the entry of Ca²⁺ into hair cells. On the other hand, Ca-ATPase and the Na⁺-Ca²⁺ exchanger are the main exits for Ca²⁺ from hair cells (Ikeda et al. 1992; Schulte 1993). The two main actions of Ca²⁺ in normal outer hair cells (OHCs) are to regulate the adaptation of transduction channels (Kennedy et al. 2003), and to mediate the cholinergic efferent response (Fuchs 1996; Oliver et al. 2000).

Acoustic overstimulation initially damages the hair cell stereocilia (Gillespie and Walker 2001) and eventually leads to hair cell death (Ou et al. 2000) and irreversible deafness (Nadol 1993). Elevation of the cytoplasmic Ca²⁺ concentration is suggested to be one of the causes of hair cell damage (Fridberger et al. 1998). The sustained elevation of cytoplasmic Ca²⁺ activates numerous Ca²⁺-dependent enzymes (Trump and Bereszky 1992; Orrenius et al. 1992). The excessive activation of these enzymes damages cells, and in turn, leads to the generation of toxic products such as free radicals that cause lethal alterations in cytoskeletal organization.

This study was designed to determine the effects of L- and T-type VGCC blockers on acoustic injury of the cochlea. Auditory brainstem response (ABR) was used to evaluate the hearing ability. ABR is an electrical signal evoked from the brainstem by the presentation of the sound. Hearing thresholds can be evaluated at each frequency using ABR. Benzodiazepines (diltiazem), phenylalkylamines (verapamil), and dihydropyridines (nicardipine and nimodipine) were used as the L-type VGCC blockers. Two T-type VGCC blockers (mibefradil and flunarizine) were also tested. We did not examine the effects of VGCC blockers of N-, P/Q-, or R-types, because they are either toxic to mammals or are not suitable for systemic injection.

Materials and Methods

Animals

Eighty-five female ddY mice (8 weeks of age) were used. The
animals were anesthetized via an intraperitoneal injection of 50 mg/kg of pentobarbital sodium (Dainihon Pharmaceuticals Inc., Osaka, Japan). Animal experiments were carried out in a humane manner after receiving approval from the Institutional Animal Experiment Committee of the University of Tsukuba and in accordance with the regulations on animal experiments of our university and the guidelines for the proper conduct of animal experiments and related activities in academic research institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Auditory brainstem response (ABR) testing

The hearing thresholds of mice were assessed according to auditory brainstem response (ABR). Needle electrodes were placed subcutaneously at the vertex, in the right retroauricular region, and in the pre-sacral region of each mouse’s brain. Tone bursts of 4-16 kHz (rise/fall time; 1 msec, and duration: 10 msec) were used for ABR testing. The evoked responses were summed and filtered with a band-pass of 200 Hz to 3 kHz using a SYNAX 1200 system (NEC, Tokyo, Japan). The thresholds were determined visually in 5-dB steps.

Histological evaluation

At the end of the study period, the mice were sacrificed under deep anesthesia. The cochleas were fixed with 4% paraformaldehyde for 8 hours. After fixation, the organs of Corti were dissected for surface preparation. The nuclei of hair cells were stained with 5 μg/ml of propidium iodide (Molecular Probes Inc., OR, USA) for 30 min at room temperature. All specimens were thoroughly inspected under a laser confocal microscope (TCS 4D, Leica Microsystems, Wetzlar, Germany) (Uemaetomari et al. 2005). Missing hair cells, i.e., the absence of propidium iodide staining, was evaluated in each animal, and the findings were compared between the drug-treated and control groups. The percentage of hair cell loss was calculated by counting 50 hair cells in each row of hair cells in a region 3.66 mm from the apex (Uemaetomari et al. 2005; Tabuchi et al. 2005; Tabuchi et al. 2006).

Acoustic overexposure

The animals were exposed to a 4-kHz pure tone of 128 dB SPL (sound pressure level) for 4 hours in an open field system while awake (Uemaetomari et al. 2005; Murashita et al. 2006; Hoshino et al. 2008).

VGCC blockers

The VGCC blockers were dissolved in 0.1 ml of physiological saline solution and were then administered intraperitoneally immediately before acoustic overexposure. The drugs and the dosages tested in this study were as follows:

(1) Diltiazem-treated group (n = 10)

- The animals were treated with 60 (n = 5) or 200 mg/kg (n = 5) of diltiazem hydrochloride (Sigma, St. Louis, MO, USA).

(2) Verapamil-treated group (n = 15)

- The animals were treated with 10 (n = 5), 30 (n = 5), or 60 mg/kg (n = 5) of verapamil hydrochloride (Sigma, St. Louis, MO, USA).

(3) Nicardipine-treated group (n = 15)

- The animals were treated with 30 (n = 5), 60 (n = 5), or 90 mg/kg (n = 5) of nicardipine hydrochloride (Sigma, St. Louis, MO, USA).

(4) Nimodipine-treated group (n = 20)

- The animals were treated with 30 (n = 5), 60 (n = 5), 120 (n = 5), or 240 mg/kg (n = 5) of nimodipine (Sigma, St. Louis, MO, USA).

(5) Mibefradil-treated group (n = 10)

- The animals were treated with 30 (n = 5) or 60 mg/kg (n = 5) of mibefradil dihydrochloride (Sigma, St. Louis, MO, USA).

(6) Flunarizine-treated group (n = 10)

- The animals were treated with 60 (n = 5) or 200 mg/kg (n = 5) of flunarizine dihydrochloride (Sigma, St. Louis, MO, USA).

(7) Control group (n = 5)

- The animals were treated with 0.1 ml of physiological saline solution.

The effects of higher dosages of these agents on acoustic injury were not examined because some of the animals were dead by administration of the higher dosages.

Data analysis

All results are presented as the mean ± s.d. The ABR threshold shift and hair cell loss were assessed with two-way ANOVA and Fisher’s PLSD test using StatView J5.0 (HULINKS, Tokyo, Japan). P-values less than 0.05 were considered to be significant.

Results

ABR studies

Fig. 1 shows the time course of the ABR threshold shift. There was no statistically significant difference in the ABR threshold shift between at one week and at two weeks (two-way ANOVA). In addition, the time course of the ABR threshold shift was essentially same among frequencies tested. Therefore, only the dose-response data of each reagent at two weeks after acoustic overexposure is shown in Table 1. Application of diltiazem at 200 mg/kg but not at 60 mg/kg significantly decreased the ABR threshold shifts one and two weeks after acoustic overexposure (Table 1, two-way ANOVA and Fisher’s PLSD test: *p < 0.05 in the 200 mg/kg subgroup). Verapamil at 60 mg/kg also decreased the ABR threshold shifts one and two weeks after acoustic overexposure, although verapamil at lower concentrations showed no apparent protective effect (**p < 0.01 in the 60-mg/kg subgroup). Nicardipine at 90 mg/kg and nimodipine at 240 mg/kg alleviated acoustic injury as compared with the control group (*p < 0.05 in the 90-mg/kg nicardipine subgroup and **p < 0.01 in the 240-mg/kg nimodipine subgroup). In contrast, the administration of mibefradil or flunarizine, each of which is a T-type VGCC blocker, did not affect the ABR threshold shift after acoustic overexposure (Table 1).

Morphological studies

The mice were sacrificed two weeks after acoustic overexposure. The first row of OHCs showed the greatest damage among the all groups of hair cells, followed by the second row of OHCs, while damage to the third row of OHCs and inner hair cells (IHCs) was limited (Fig. 2a). Administration of a L-type VGCC blocker, diltiazem (200 mg/kg), verapamil (60 mg/kg), nicardipine (90 mg/kg), or nimodipine (240 mg/kg), significantly decreased the loss of OHCs (Fig. 2b, two-way ANOVA and Fisher’s PLSD test: *p < 0.05). However, administration of a T-type VGCC blocker, i.e., mibefradil (60 mg/kg) or flunarizine (200 mg/
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Fig. 1. Time courses of ABR threshold shifts in the mice treated with L-type VGCC blockers. The animals were treated with each L-type VGCC blocker and then subjected to acoustic overexposure of 128 dB SPL for 4 hours. ABR threshold shifts at one and two weeks after acoustic overexposure are shown. There was no statistically significant difference in the ABR threshold shifts between one week and at two weeks (two-way ANOVA).

Table 1. The effects of L- and T-type VGCC blockers on acoustic injury.

<table>
<thead>
<tr>
<th>Drug</th>
<th>4 kHz</th>
<th>6 kHz</th>
<th>8 kHz</th>
<th>16 kHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>41.0 ± 7.4</td>
<td>40.0 ± 6.1</td>
<td>37.0 ± 4.5</td>
<td>46.0 ± 16.4</td>
</tr>
<tr>
<td>diltiazem</td>
<td>60 mg/kg</td>
<td>37.0 ± 19.9</td>
<td>37.0 ± 14.4</td>
<td>44.0 ± 9.6</td>
</tr>
<tr>
<td></td>
<td>200 mg/kg*</td>
<td>20.0 ± 12.2</td>
<td>31.0 ± 6.3</td>
<td>36.0 ± 10.3</td>
</tr>
<tr>
<td>nicardipine</td>
<td>30 mg/kg</td>
<td>37.0 ± 4.5</td>
<td>40.0 ± 5.0</td>
<td>37.0 ± 11.5</td>
</tr>
<tr>
<td></td>
<td>60 mg/kg</td>
<td>45.0 ± 8.2</td>
<td>41.0 ± 7.4</td>
<td>42.0 ± 7.6</td>
</tr>
<tr>
<td></td>
<td>90 mg/kg*</td>
<td>26.0 ± 17.5</td>
<td>31.0 ± 18.2</td>
<td>36.0 ± 22.2</td>
</tr>
<tr>
<td>nimodipine</td>
<td>30 mg/kg</td>
<td>24.0 ± 11.4</td>
<td>35.0 ± 7.9</td>
<td>38.0 ± 12.6</td>
</tr>
<tr>
<td></td>
<td>60 mg/kg</td>
<td>27.0 ± 14.4</td>
<td>35.0 ± 9.4</td>
<td>41.0 ± 9.6</td>
</tr>
<tr>
<td></td>
<td>120 mg/kg</td>
<td>33.0 ± 15.2</td>
<td>43.0 ± 15.2</td>
<td>40.0 ± 15.0</td>
</tr>
<tr>
<td></td>
<td>240 mg/kg**</td>
<td>20.0 ± 18.7</td>
<td>26.0 ± 19.9</td>
<td>32.0 ± 9.7</td>
</tr>
<tr>
<td>verapamil</td>
<td>10 mg/kg</td>
<td>32.0 ± 7.6</td>
<td>41.0 ± 4.2</td>
<td>42.0 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>35.0 ± 11.2</td>
<td>37.0 ± 16.0</td>
<td>40.0 ± 12.7</td>
</tr>
<tr>
<td></td>
<td>60 mg/kg*</td>
<td>26.0 ± 6.5</td>
<td>33.0 ± 5.7</td>
<td>31.0 ± 12.9</td>
</tr>
<tr>
<td>mibefradil</td>
<td>30 mg/kg</td>
<td>36.0 ± 12.6</td>
<td>43.0 ± 13.5</td>
<td>40.0 ± 11.4</td>
</tr>
<tr>
<td></td>
<td>60 mg/kg</td>
<td>38.0 ± 12.5</td>
<td>46.0 ± 10.4</td>
<td>42.0 ± 11.4</td>
</tr>
<tr>
<td>flunarizine</td>
<td>60 mg/kg</td>
<td>32.0 ± 9.5</td>
<td>40.0 ± 14.4</td>
<td>42.0 ± 7.5</td>
</tr>
<tr>
<td></td>
<td>200 mg/kg</td>
<td>35.0 ± 24.2</td>
<td>44.0 ± 21.7</td>
<td>47.0 ± 20.2</td>
</tr>
</tbody>
</table>

ABR threshold shifts at two weeks after acoustic overexposure are shown. Treatment with diltiazem (200 mg/kg), nicardipine (90 mg/kg), nimodipine (240 mg/kg) or verapamil (60 mg/kg) significantly decreased the ABR threshold shift (two-way ANOVA and Fisher’s PLSD test: *p < 0.05 and **p < 0.01 as compared with the control group).
VGCCs are divided into L, N, P/Q, R, and T-types according to their electrophysiological and pharmacological properties (Triggle 1999). L-type VGCCs are blocked by benzodiazepines, phenylalkylamines, and dihydropyridines (Nowycky et al. 1985). P/Q-type VGCCs are blocked by \( \omega \)-agatoxin-IVA (Mintz et al. 1992). N-type VGCCs are selectively and irreversibly blocked by \( \omega \)-conotoxin-GVIA (Kerr and Yoshikami 1984). Blockage of the R-type VGCC current is achieved by SNX-482 (Tottene et al. 2000). Mibefradil selectively blocks T-type VGCC (Martin et al. 2000). In this study, we examined the effects of blockers of L- and T-type VGCC, because the other types of VGCC blockers are either toxic to mammals or are not suitable for systemic injection.

Elevation of the cytoplasmic Ca\(^{2+}\) concentration was observed in the OHCs during acoustic overstimulation (Fridberger et al. 1998). Sustained elevation of cytoplasmic Ca\(^{2+}\) is thought to damage OHCs by activating numerous Ca\(^{2+}\)-dependent enzymes (Trump and Berezesky 1992), including calpains and other proteases, protein kinases, nitric oxide synthase, calcineurins, and endonucleases. Because the excessive activation of these enzymes leads to the generation of toxic products such as free radicals or lethal alterations in cytoskeletal organization, regulation of the activities of Ca\(^{2+}\)-dependent enzymes is essential for cell survival in cochlear injuries.

As for L-type VGCC blockers, only the effects of diltiazem on acoustic cochlea injuries have been examined, but the role of diltiazem remains controversial. Some guinea pig studies showed that diltiazem had a protective effect.
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against acoustic injury (Heinrich et al. 1999; Maurer et al. 1999) and showed a tendency to reduce the acoustic trauma caused by drilling during otologic surgery in humans (Maurer et al. 1995). On the other hand, Boettcher reported that diltiazem did not protect the cochlea from noise-induced hearing loss in gerbils (Boettcher 1996). We included three types of L-type VGCC blockers in this study to determine the effect of L-type VGCC blockers on acoustic injuries. The present findings show that various L-type VGCC blockers have protective effects against acoustic injury of the cochlea. The protective effects of verapamil against kanamycin-induced ototoxicity have also been reported (Zburavskii et al. 2002).

The localization of VGCC has been documented in the cochlea of the mouse (Hafidi and Dunol 2004), guinea pig (Layton et al. 2005), and chinchilla (Lopez et al. 2003). In the mouse cochlea, immunohistological staining for L-type VGCC was observed broadly in the stria vascularis (SV), spiral ligament, spiral limbus, organ of Corti (supporting cells and inner and outer hair cells), and spiral ganglion neurons. Staining for T-type VGCC was also observed in the SV, organ of Corti (supporting cells and inner and outer hair cells), and neurons (Hafidi and Dunol 2004; Shen et al. 2007). The VGCC expressed in mammalian hair cells are predominantly Cav1.3 (α1D) subunits of L-type Ca\(^{2+}\) channels. L-type VGCC currents in auditory hair cells are believed to be carried by Cav1.3 (α1D) subunit channels (Brandt et al. 2003). Furthermore, this Cav1.3 (α1D) subunit is essential for cochlear development (Hafidi and Dunol 2004) and for regulation of the expression of large-conductance Ca\(^{2+}\)-activated potassium and small-conductance Ca\(^{2+}\)-activated potassium channels (Nemzou et al. 2006). The absence of Cav1.3 channels causes deafness and the degeneration of developing OHs and IHs in mutant mice (Platzer et al. 2000; Glueckert et al. 2003).

L-type VGCC mediate Ca\(^{2+}\) currents in hair cells among many species, such as the turtle (Art et al. 1986), chick (Fuchs and Evans 1990; Kollmar et al. 1997; Spassova et al. 2001), guinea pig (Nakagawa et al. 1991; Chen et al. 1995), and mouse (Zhang et al. 1999; Platzer et al. 2000; Engel et al. 2002). The effects of L-type VGCC blockers on cochlear-evoked potential have been reported. Cochlear perfusion of nimodipine results in reversible, dose-related suppression of the compound action potential, reduced cochlear microphonics, and a negative summating potential. The endocochlear potential is not affected by nimodipine (Bobbin et al. 1991; Zhang et al. 1999). Sueta et al. (2004) suggested that L-type VGCC presynaptically regulate evoked and spontaneous neurotransmitter release from hair cells. Spontaneous neural noise is also reversibly suppressed by nimodipine. These findings suggest that the L-type VGCC and Cav1.3 subunits are important for mediating neurotransmitter release as well as the physiological maintenance of hair cells.

In contrast to the protective effect of L-type VGCC blockers, T-type VGCC blockers, i.e., mibebradil and flunarizine, did not exert any protective effect in this study. The family of T-type calcium channels is composed of three members (Ca\(^{3+}\), Ca\(^{3+}\), and Ca\(^{3+}\)) based on their respective main pore-forming alpha subunits: α1G, α1H, or α1I. Immunohistochemical and PCR studies have demonstrated that Ca\(^{3+}\) and Ca\(^{3+}\) are expressed in the cochlea (Shen et al. 2007). Although the T-type VGCC blockers tested in the present study did not exert protective effects, Shen et al. (2007) reported the prophylactic and therapeutic effects of other T-type VGCC blockers, such as ethosuximide and trimethadione, on acoustic injury of the mouse. One possible explanation for the disagreement in the results may be the differences in the route and duration of administration of T-type VGCC blockers. Trimethadione and ethosuximide were orally administered for two weeks in the study of Shen et al. (2007), whereas we administered mibebradil and flunarizine intraperitoneally once after acoustic overexposure. There is a possibility that mibebradil and flunarizine may exhibit protective effects against acoustic injury in long-term administration. Another difference was the method that generates acoustic overexposure. Acoustic injury was induced by 4-kHz pure tone of 128 dB SPL for 4 hours in our study, while mice were subjected to noise exposure of 110 dB SPL for 30 min in the study of Shen et al. (2007). Finally, selectivity of T-type VGCC blockers may account for the protective effects of trimethadione and ethosuximide. Recent studies have suggested that the blocking of ionic channels other than T-type VGCC is relevant to the antiepileptic actions of ethosuximide because most studies failed to confirm the blocking of T-type currents at therapeutically relevant concentrations of ethosuximide (Heady et al. 2001). Unfortunately, no compounds that are selective for T-type VGCC have been identified so far; thus, it is uncertain whether the selective inhibition of T-type VGCC is of clinical importance in acoustic injury. Unquestionably, further experiments are needed to determine the role of T-type VGCC in cochlear acoustic injury.

In conclusion, L-type VGCC blockers showed significant protective effects against acoustic injury in the cochlea of mice. Our results may promote development of preventative strategy against acoustic injury of the cochlea.

Acknowledgment

This work was supported by Grants-in-aid for Scientific Research (C) 20591969 and for Young Scientists (B) 18791186 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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