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INHIBITION OF THE CALCIUM- AND VOLTAGE-DEPENDENT BIG CONDUCTANCE POTASSIUM CHANNEL AMELIORATES CISPLATIN-INDUCED APOPTOSIS IN SPIRAL LIGAMENT FIBROCYTES OF THE COCHLEA

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Abstract—The role of calcium- and voltage-dependent big conductance potassium channels in regulating apoptosis was investigated in cultured type I spiral ligament fibrocytes. Incubation of type I spiral ligament fibrocytes derived from gerbil cochlea with cisplatin induced dose- and time-dependent apoptosis as demonstrated by annexin V conjugated to fluorescein isothiocyanate/prodidium iodide assays. The average voltage activation threshold of whole cell current was sharply shifted to −40 mV in the cisplatin-treated cells as compared with a value of 40 mV in control cells. The average whole-cell current of cisplatin-treated cells induced by a depolarization voltage step from −80 to −10 mV was increased significantly to 1.2±0.4 nA as compared with 0.08±0.1 nA in control cells. Coincubation with tetraethylammonium and cisplatin retained the whole cell current in the normal range (0.12±0.2 nA). The increment of cisplatin-induced whole-cell current was inhibited (97±5%) by a specific calcium- and voltage-dependent big conductance potassium channel blocker iberiotoxin. Consistent with this, co-incubation with tetraethylammonium significantly attenuated cisplatin-induced apoptosis in type I spiral ligament fibrocytes by more than 50%. We conclude that the activation of BK channels is an early event associated with cisplatin-induced apoptosis in type I spiral ligament fibrocytes. These findings also point to the calcium- and voltage-dependent big conductance potassium channels as a potential pharmacological target for manipulating cisplatin ototoxicity.

Key words: apoptosis, cisplatin, gerbil, inner ear, K channel, ototoxicity.

Cisplatin ototoxicity has been well documented in both clinical and animal studies. When systemically administrated, it can cause a progressive, irreversible, bilateral sensorineural hearing loss and tinnitus (Helson et al., 1978; Von Hoff et al., 1979). Vestibular impairments have also been reported, although less frequently (Schaefer et al., 1981; Black et al., 1982). Hearing loss usually affects high frequencies first and may gradually spread to lower frequencies involved in speech recognition (Waters et al., 1991; Simon et al., 2002).

Recent results from both *in vitro* and *in vivo* experiments have shown that apoptotic cell death is the primary cause of cisplatin toxicity (Boulikas and Vougiouka, 2003). Animal models for cisplatin ototoxicity have shown dose-dependent damages in sensory hair cells, ganglion neurons and non-neuronal cells of the inner ear (Sergi et al., 2003; Meech et al., 1998; Alam et al., 2000). In the cochlea, cisplatin has been shown by TUNEL analysis and morphological apoptotic landmarks to induce apoptosis in hair cells, supporting cells, spiral ganglion neurons (SGNs) and stria vascularis cells (Alam et al., 2000). Cisplatin-induced activation of caspases has also been reported in cochlear tissue both *in vitro* and *in vivo* (Liu et al., 1998; Kalinec et al., 2003; Devarajan et al., 2002). In addition, cisplatin promoted the up-regulation of the pro-apoptotic tumor suppresser gene p53, a significant increase in bax-positive and decrease in bcl-2-positive cells and the release of cytochrome c (Alam et al., 2000; Devarajan et al., 2002; Lee et al., 2003; Zhang et al., 2003).

In contrast to the cell swelling observed during necrosis, cell shrinkage is recognized as a morphological hallmark during early stages of apoptosis (Kerr et al., 1972). Recent reports have linked the activity of K channels to apoptosis in a variety of model systems. Evidence is accruing that loss of intracellular K^+ is responsible for the apoptotic cell shrinkage that ultimately leads to death in various epithelial cells, thymocytes, fibroblasts, lymphocytes, cortical neurons and smooth muscle cells (Gomez-Angelats and Cidlowski, 2002; Gomez-Angelats et al., 2000; Yu, 2003). Voltage-dependent K channels play a critical role in mediating the K^+ efflux associated with apoptotic cell shrinkage, and blockage of K channel activity has been shown to reduce and/or prevent apoptosis (Yu, 2003).

Although apoptosis has been directly linked to cisplatin ototoxicity, the role of K channels in mediating cisplatin-induced apoptosis has not been studied. We have derived a secondary cell culture line from the spiral ligament fibrocytes (SLFs) of gerbil cochlea and characterized the cells as type I SLFs based on their morphological and immuno-

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Abbreviations: annexin V-FITC, annexin V conjugated to fluorescein isothiocyanate; BK channel, calcium- and voltage-dependent big conductance potassium channel; EP, endocochlear potential; α-MEM, α-minimum essential medium; PBS, phosphate-buffered saline; SLFs, spiral ligament fibrocytes; TEA, tetraethylammonium.

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histochemical properties (Liang et al., 2003). We have shown that the membrane ionic conductance of these cells is dominated by a voltage- and Ca^{2+}-dependent big conductance K channel (BK channel) (Liang et al., 2003; Shen et al., 2004). In this study, we report that cisplatin-induced apoptosis in type I SLFs involves activation of the BK channels. Furthermore, blocking the activation of BK channels ameliorates the cisplatin-induced apoptosis.

EXPERIMENTAL PROCEDURES

Cell cultures

Ten young adult Mongolia gerbils of both genders were used for harvesting cochlear spiral ligament tissues for cell culture. Animals were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed in the rodent facility of the Medical University of South Carolina. Animals were killed under anesthesia (urethane, 1.5 g/kg, i.p.), and both temporal bones were removed rapidly and transferred to a glass microdissection dish filled with ice-cold a-minimum essential medium (α-MEM) culture media. The cochlear spiral ligament was microdissected under aseptic conditions. The protocol for use and handling of animals complied with federal regulations, the Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee (IACUC) of the Medical University of South Carolina.

Methods used for establishing the secondary cell cultures of type I SLFs have been described in detail previously (Liang et al., 2003; Shen et al., 2004). Briefly, the coiled spiral ligament was diced into small fragments and explanted in 35 mm Petri dishes (one cochlea/dish) for primary culture. Petri dishes were kept in a CO_2 incubator at 37 °C with maximal humidity and fed twice a week. Upon reaching full confluence, type I SLFs were subcultured. Cells from passages 3–10 were used for the experiments. The culture media consists of α-MEM supplemented with hydrocortisone (36 ng/ml), insulin (5 μg/ml), selenium (5 ng/ml), transferrin (5 μg/ml), triiodothyronine (4 pg/ml), 0.1% penicillin and 10% fetal bovine serum, all purchased from Gibco Chemical Co. (St. Louis, MO, USA).

Quantitative analysis of apoptosis

Type I SLFs were seeded into 60 mm culture dishes and grown in a CO_2 incubator with maximum humidity for 2–3 days to reach sub-confluence before cisplatin treatment. Cisplatin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) to obtain a 20 mM stock solution. The cisplatin stock solution was mixed directly with the culture media to reach the final concentration of 5, 10, 20, 40 or 80 μm and incubated with the cells for up to 72 h. Cells were harvested at the end of each incubation period and subjected to various assays.

Annexin V-FITC and Propidium Iodide (PI) were used to evaluate apoptosis in type I SLFs (Barriere et al., 2001). Annexin V conjugated to fluorescein isothiocyanate (annexin V-FITC) binds the phosphatidyl-serine translocated to the outer surface of the plasma membrane during early apoptosis and PI binds to the nucleus of cells with a damaged and leaky plasma membrane (Vermes et al., 1995). Cells in the early stages of apoptosis are annexin V-FITC positive and in the later stages of apoptosis are double labeled by both annexin V-FITC and PI. Dead cells are positive only for PI.

For fluorescent microscopy studies, type I SLFs were gown to confluence in glass slide chamber and incubated for various time periods with different doses of cisplatin. At the end of the incubation period, cells were washed twice using the binding buffer (10 mM HEPES–NaCl, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) provided with the annexin V-FITC/PI kit (Sigma-Aldrich) and exposed to annexin V-FITC and PI in the dark at room temperature for 15 min. The control and treated cells were washed twice with phosphate-buffered saline (PBS) and immediately examined and photographed using a fluorescent microscope (Zeiss).

Quantitative analysis of cisplatin-induced apoptosis in the type I SLFs was performed using the annexin V-FITC/PI assay and flow cytometry. Cisplatin-treated and untreated cells were harvested and washed twice in PBS (pH 7.4). Around $\sim 1\times10^5$ cells obtained from each dish were suspended in 500 μl of binding buffer, 5 μl of annexin V-FITC (50 μg/ml) and 10 μl of PI (100 μg/ml). After incubation in the dark for 10 min at room temperature, the samples were analyzed using a Becton Dickinson flow cytometry and CellQuest software (Becton Dickinson, CA, USA). The cells were apportioned into four groups including: (1) alive, (2) early apoptosis, (3) late apoptosis and (4) dead based on their staining pattern with annexin V-FITC and PI. A total of $\sim 1\times10^4$ events were gated for each sample and each group was expressed as a percentage of the total events. Each experiment was repeated at least three times and an average percentage of each group was obtained for statistical analysis using the unpaired Student's *t*-test.

Cell electrophysiology

Type I SLFs were washed in PBS and treated with 0.05% trypsin/EGTA at room temperature for 3–5 min. Cells were harvested and suspended in culture media in a 15 ml centrifuge tube and kept in a CO_2-incubator for up to 3 h for electro-physiological experiments. For physiological recordings, a small number of cells were transferred by micropipette to a recording chamber mounted on an inverted microscope and allowed $\sim$2 min to settle in the chamber. Single cells were easily identified and patched under the microscope. The recording chamber was superfused with bath solution at 0.5 ml/min (chamber volume: 40 μl).

Recording pipettes were manufactured from Corning 7052 glass tubes using a programmable horizontal puller (Sutter, P-97, CA, USA). The tips of the recording pipette were heat polished with a microforge to form an opening around 1 μm (I.D.). A perforated whole-cell configuration was obtained with amphotericin B in the pipette solution (Shen et al., 1997). The average access resistance and membrane capacitance was 48.1 ± 7.1 MΩ and 11.7 ± 5.0 pF, respectively ($n=26$), and $\sim$80% compensation was used for all recordings. Data were collected via an Axon 200B patch clamp amplifier and an Axon digidata-1200 A/D converter integrated with a Pentium II PC. Software Axon pClamp 8.0, Microcal Origin 5.0 and Microsoft Excel were used for data acquisition and analysis. Paired and unpaired Student's *t*-tests were used for statistical analysis.

The pipette solution contained (in mM): 150 KCl, 1 Mg-ATP, 0.4 $CaCl_2$, 1 EGTA, 10 HEPES (pH 7.4). The bath solution contained (in mM): 145 NaCl, 4.5 KCl, 1 $MgCl_2$, 0.7 $CaCl_2$, 10 HEPES (pH 7.4). Cisplatin, tetraethylammonium (TEA), iberiotoxin and nifedipine were prepared just prior to applications. All drugs and chemicals used for electro-physiological studies were purchased from Sigma-Aldrich.

RESULTS

The cisplatin concentrations used here are comparable to those used for inducing apoptosis in inner ear hair cells and spiral ganglion neurons *in vitro* (Zhang et al., 2003; Chen et al., 2001). Fig. 1A shows a typical phase contrast image of fully confluent type I SLFs in culture. Untreated cells incubated with annexin V-FITC and PI showed only low background fluorescence (Fig. 1B). Incubation with 40 μM cisplatin for 48 h resulted in a substantial increase in cells stained positive for annexin V-FITC along with a