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Vanilloid Receptors in Hearing: Altered Cochlear Sensitivity by Vanilloids and Expression of TRPV1 in the Organ of Corti

Jiefu Zheng[1], **Chunfu Dai**[1,2], **Peter S. Steyger**[1], **Youngki Kim**[3], **Zoltan Vass**[4], **Tianying Ren**[1], and **Alfred L. Nuttall**[1,5]

[1]Oregon Hearing Research Center, Department of Otolaryngology/Head and Neck Surgery, Oregon Health & Science University, Portland, Oregon 97239

[2]Department of Otolaryngology, Eye Ear Nose and Throat Hospital, Fudan University, Shanghai, 200031, People's Republic of China

[3]Department of Otolaryngology, Chonbuk National University Medical School, Chonju, Chonbuk, 561-712 Korea

[4]Department of Otolaryngology, Albert Szent-Gyorgyi Medical University, H-6724 Szeged, Hungary

[5]Kresge Hearing Research Institute, The University of Michigan, Ann Arbor, Michigan 48109-0506

Abstract

Capsaicin, the vanilloid that selectively activates vanilloid receptors (VRs) on sensory neurons for noxious perception, has been reported to increase cochlear blood flow (CBF). VR-related receptors have also been found in the inner ear. This study aims to address the question as to whether VRs exist in the organ of Corti and play a role in cochlear physiology. Capsaicin or the more potent VR agonist, resiniferatoxin (RTX), was infused into the scala tympani of guinea pig cochlea, and their effects on cochlear sensitivity were investigated. Capsaicin (20 μM) elevated the threshold of auditory nerve compound action potential and reduced the magnitude of cochlear microphonic and electrically evoked otoacoustic emissions. These effects were reversible and could be blocked by a competitive antagonist, capsazepine. Application of 2 μM RTX resulted in cochlear sensitivity alterations similar to that by capsaicin, which could also be blocked by capsazepine. A desensitization phenomenon was observed in the case of prolonged perfusion with either capsaicin or RTX. Brief increase of CBF by capsaicin was confirmed, and the endocochlear potential was not decreased. Basilar membrane velocity (BM) growth functions near the best frequency and BM tuning were altered by capsaicin. Immunohistochemistry study revealed the presence of vanilloid receptor type 1 of the transient receptor potential channel family in the hair cells and supporting cells of the organ of Corti and the spiral ganglion cells of the cochlea. The results indicate that the main action of capsaicin is on outer hair cells and suggest that VRs in the cochlea play a role in cochlear homeostasis.

INTRODUCTION

In mammals, vanilloid-sensitive neurons can be excited by a variety of noxious factors such as chemical, mechanical, or thermal stimuli and transmit nociceptive information from the

Address for reprint requests: A. L. Nuttall, Oregon Hearing Research Center, Oregon Health & Science University, 3181 SW Sam Jackson Park Rd., NRC04, Portland, OR 97239 (nuttall@ohsu.edu)..

periphery into the CNS, resulting in a sensation of burning pain. These neurons, called nociceptors, are characterized in part by their sensitivity to capsaicin, the pungent ingredient in “hot” chili peppers (Caterina et al. 1997; Szallasi and Blumberg 1999). Capsaicin exerts its effect on nociceptors by selectively activating vanilloid receptors (VRs), the ligand-gated nonselective cation channels, leading to membrane depolarization and action potential generation. In addition, the activation of VRs in peripheral vanilloid-sensitive nerve endings is responsible for neurogenic inflammation that is characterized by vasodilation and vascular permeability increase (Kress et al. 1997; Szallasi and Blumberg 1999; Vyklicky et al. 1998). A subtype of VR, called VR1 (now named TRPV1. TRP stands for transient receptor potential channel) (Montell et al. 2002), has been cloned and shown to be expressed in a subset of sensory neurons in the dorsal root (DRG) and trigeminal ganglia (TG) (Caterina et al. 1997). The expression of TRPV1 is not restricted to primary sensory neurons. Instead, it is reported that TRPV1 and TRPV2 (formerly named VRL-1, VR-like protein) receptors are widely expressed in the brain and some nonneuronal tissues, which places VRs in a much broader perspective than pain perception (Mezey et al. 2000; Minke and Cook 2002; Sasamura et al. 1998; Szallasi and Blumberg 1999). Moreover, the expression of a VR-related osmotically activated channel has been demonstrated in mouse inner ear (i.e., the inner and outer hair cells and stria vascularis) as well as in neurosensory cells in the CNS responsible for systemic osmotic pressure, TG, and Merkel cells (Liedtke et al. 2000). In cochlear physiology studies, it has been reported that capsaicin regulates guinea pig cochlear blood flow via substance P (SP) and nitric oxide (NO)-mediated mechanism (Vass et al. 1994– 1996). However, the existence and distribution of VRs in the organ of Corti remain unknown as is whether VRs may play any role in hearing sensitivity if they do exist in the cochlea. Here, we report that vanilloids can regulate cochlear sensitivity and that TRPV1 receptors are present in the organ of Corti. Some of the preliminary data of this study have been presented (Zheng et al. 2001a).

METHODS

Cochlear physiological experiments

In mammalian cochlea, auditory signal transduction is accomplished by concerted action of the inner and outer hair cells (IHCs and OHCs) in the organ of Corti. The IHCs detect the acoustic signal and transfer it into action potentials of the auditory nerve. OHCs amplify the sound-evoked motion of the basilar membrane (BM) through an active feed-back mechanism that assures the high sensitivity, sharp tuning, and large dynamic range for acoustic signal processing in the cochlea. Cochlear sensitivity is also determined by other factors including the blood supply, and the electrical and ionic environment of the cochlear fluids. In this study, we investigated the effects of capsaicin and its agonist on the cochlear physiology at various aspects as described in the following text, to provide a comprehensive description of how the vanilloids act in the cochlea.

ANIMAL PREPARATION—Thirty-six pigmented guinea pigs (strain 2NCR, obtained from the Charles River Laboratory) with Preyer’s reflex weighing 250–350 g were used in this study for physiological experiments. The animals were housed in American Association for Accreditation of Laboratory Animal Care approved facilities. Experimental protocols were approved by the Committee on the Use and Care of Animals, Oregon Health & Science University. The animals were anesthetized using both ketamine (40 mg/kg im) and xylazine (10 mg/kg im). Supplemental doses of ketamine and xylazine were given on a schedule or as needed, judging by leg withdrawal to a toe pinch.

Rectal temperature of the animals was maintained at 38 ± 1°C with a servo-regulated heating blanket. Cochlear temperature was additionally controlled by supplemental heat to

the head from a lamp and a heated head-holder. The electrocardiogram and heart rate were continuously monitored as measures of anesthesia level and the general condition of the animal. All presented data were collected from animals with normal electrocardiograms and with heart rate between 270 and 380 beats/min.

The guinea pig's head was firmly fixed in a heated head-holder, which was mounted on a custom-made manipulator and electrically isolated from the operation table. A tracheotomy was performed, and a ventilation tube was inserted into the trachea to insure free breathing. A ventral and postauricular combined approach was used to expose the left auditory bulla with a large part of the external ear being removed, while the medial 2/3 of the external auditory canal was preserved to facilitate placement of the acoustic speculum. The bulla was widely opened to expose the cochlea. The middle ear muscle tendons were carefully sectioned.

MEASUREMENT OF COCHLEAR POTENTIALS—Compound action potential (CAP) and cochlear microphonic (CM) are sound-evoked cochlear potentials used for cochlear function assessment. The CAP is the action potential produced by synchronized auditory nerve firing in response to brief acoustic stimuli and is used to assess overall cochlear sensitivity at different frequencies. In contrast, the CM is an AC receptor potential produced primarily by the OHCs of the organ of Corti during acoustic stimulation. For CAP and CM measurement, a ball electrode made of Teflon-coated silver wire (75 μm in diameter) was placed in the round window niche and fixed on the bulla with carboxylate cement. An Ag/AgCl wire was inserted into neck soft tissue medial to the exposed bulla to serve as the ground electrode. A plastic three-way coupler with two speakers (made of ½-in B&K microphones) and an Etymotic 10 B^+ microphone were fitted to the ear canal to deliver acoustic stimuli and record the otoacoustic emissions (OAEs). Tone bursts (10 ms in duration, 1-ms rise/fall) were generated using a 16-bit D/A converter (Tucker Davis Technologies) and delivered to the ear canal as acoustic stimuli to evoke the CAP and CM. The round window signal was amplified 1,000 times by an AC preamplifier (Grass Instrument, Model P15) and a custom-designed AC amplifier. After A/D conversion and averaging, the evoked electrical responses from the round window were digitalized and saved for post processing for the CAP and CM. The amplified signal was also displayed on an oscilloscope for CAP threshold assessment to monitor the hearing sensitivity during the experiment. The N_1 detection of 10 μV without averaging was used as the CAP threshold criterion.

The electrophysiology of the IHCs and OHCs relies on the normal level of the endocochlear potential (EP), a large positive resting DC potential in the scala media of the cochlea duct, which is generated and maintained by the normal function of the stria vascularis, and integrity of the structure of the scala media (i.e., the perilymph-endolymph barrier). For EP recording, a small hole was carefully drilled in the cochlear bony wall of the second turn. A glass micro-electrode (tip diameter: ~0.5 μm) filled with 150 mM KCl and held on a micromanipulator was inserted into the scala media through the spiral ligament and stria vascularis. An Ag/AgCl wire was inserted into neck muscles to serve as the ground electrode. The DC potential was measured with an amplifier (BMA 200 AC/DC Bioamplifier, CWE) and recorded with a computerized chart recorder.

EXTRACOCHLEAR CURRENT STIMULATION AND EEOAE MEASUREMENT—Electrically evoked OAE (EEOAEs) produced by extracochlear current stimulation have been used as a noninvasive tool to investigate the in vivo electromotility of OHCs (Nuttall et al. 2001; Ren and Nuttall 1995). The method for EEOAE recording used in this study was the same as what has been described (Ren and Nuttall 1995; Zheng et al. 2001b). In brief, a Teflon-insulated platinum-iridium wire (75 μm diam) with a bare end was placed in the

round window niche for electrical stimulation. A sinusoidal signal was generated by a computer-controlled lock-in amplifier (SR830 DSP, Stanford Research Systems), and AC (35 μA RMS) was delivered to the stimulation electrode by a custom-made opto-isolated constant current stimulator. An Ag/AgCl ground electrode was inserted into the neck muscle next to the ipsilateral bulla. The EEOAE was recorded from the ear canal with a microphone (Etymotic Research ER-10B$^+$, Elk Grove Village, IL). The magnitude and phase of the output signal from the microphone preamplifier was measured at the stimulus frequency using the lock-in amplifier and was recorded with a computerized chart recorder at the sampling rate of two samples per second. Each amplitude spectrum and its corresponding phase spectrum were obtained by linearly sweeping the current from 400 Hz to 40 kHz at 50 Hz/s with a lock-in time constant of 1 s.

A recently developed multiple-component analysis method (MCA) (Ren and Nuttall 2000; Ren et al. 2000) was used to measure the multiple delays of the EEOAEs in this study. The real part of the electrically evoked OAE (X) was calculated from the amplitude (A) and phase (θ) spectra according to: $X = A\cos(\theta)$ and presented as a function of the frequency. The "delay" spectrum of X was obtained using the fast Fourier transform (FFT).

MEASUREMENT OF BM VELOCITY—To establish whether vanilloid agonists alter the cochlear mechanical responses, the magnitude and phase transfer function of BM transverse velocity was measured at the 17-kHz best-frequency location. To record the responses of the BM, a small opening was made in the first-turn scala tympani bony wall of the cochlea. Gold coated glass beads (20 μm diam) were "dropped" onto the BM (at approximately the 17-kHz place) to serve as reflective objects that track the motion of the BM. The laser beam of a laser Doppler velocimeter (Polytec, OFV 1102) was focused on a bead with the aid of a compound microscope (Nuttall et al. 1991). Sounds were presented to the external ear as described in the preceding text. The signal output of the velocimeter was digitalized at the sampling rate of 250 kHz and stored in the computer. BM velocities were determined after fast Fourier transform of the Hanning-windowed responses from the velocimeter.

COCHLEAR BLOOD FLOW MEASUREMENT—It has been reported that round window membrane application of capsaicin resulted in an increase of the cochlear blood flow (CBF) (Vass et al. 1995). This study determines the CBF change that occurs when capsaicin is directly infused into the cochlea. We also determine the relationship between the alterations of CBF and EP during capsaicin perfusion, inasmuch as the EP depends on the CBF. The CBF was measured using the laser Doppler flowmetry (LDF) technique (Miller and Nuttall 1990). Briefly, the cochlear mucosa was gently removed with a cotton pledget. The probe of the LDF (Laser Doppler System, Type PF 4001, Perimed) was placed on the lateral wall of the basal turn of the cochlea to detect the blood flow. Petroleum gel was applied to the probe tip to provide efficient laser light coupling to the cochlea. All recordings were done under stable light illumination. The change of CBF is presented as percentage value relative to preexposure level.

PERILYMPHATIC PERFUSION OF THE SCALA TYMPANI—Perilymphatic perfusion was performed to deliver chemicals into the scala tympani of the cochlea. An inlet hole (diameter: ~70 μm) was made in the scala tympani of the basal turn of the cochlea close to the round window niche and the outlet hole (diameter: ~80 μm) on the apex of the cochlea. A three-way perfusion device that allows solution substitution was used for scala tympani perfusion. A polyethylene tube was connected to this device and its fine tip (diameter: ~60 μm) was inserted into the inlet hole of the cochlea. Tissue glue was applied to seal the inlet hole and fasten the tube in position. Stock solutions were prepared as follows. Capsaicin (20 mM) was dissolved with a mix solution of ethanol (10%) and Tween-80 (10%). Resiniferatoxin (RTX; 2 mM) was dissolved in absolute ethanol.

Capsazepine (20 mM) was dissolved in absolute methanol. The preceding stock solutions were diluted with artificial perilymph [(in mM) 125 NaCl, 3.5 KCl, 25 $NaHCO_3$, 1.3 $CaCl_2$, 1.14 $MgCl_2 \cdot 6H_2O$, 0.51 $NaH_2PO_4 \cdot H_2O$, 5.0 Tris, 3.3 glucose,2.1 urea] for required concentrations just prior to use. The pH of all solutions was 7.4 and the osmolality was 300 ± 10 mOsm. Perfusates were infused into the scala tympani at a perfusion rate of 2 μl/min using a syringe pump (Sage Instruments, Model 351). The duration of perfusion for each chemical was usually 10 min but was prolonged to as long as 30 min in certain cases as needed. Artificial perilymph was infused into the cochlea in control experiments before chemical perfusion and for drug washout. The duration for drug washout was usually 30 min. Effluent from the outlet hole on the apex was absorbed within the bulla using cotton wicks.

Immunolabeling of TRPV1 receptors

Eight adult guinea pigs and 10 rats with Preyer's reflex weighing 300–350 g were used, since the available antibodies for TRPV1 immunolabeling are specific for rats. Animals were anesthetized with ketamine (100 mg/kg im) and xylazine (2 mg/kg im), and a cardiac perfusion with saline followed by 4% paraformaldehyde in 0.02 M PBS was performed. The organ of Corti and DRG (positive control) of either the guinea pig or rats were dissected and fixed in the solution of 4% paraformaldehyde in 0.02 M PBS for 3 h. The fixed tissues were washed in 0.02 M PBS (pH 7.4), permeabilized with 0.5% Triton X-100 (Sigma) for 1 h and immunoblocked in 10% goat serum in 1% bovine albumin in 0.02 M PBS for 1 h. They were then incubated with rabbit anti-TRPV1 polyclonal antibody (gift from Dr. Caterina) diluted 1:1,000 in 1% BSA-PBS for 48 h. After washing in 1% BSA-PBS, tissues were subsequently incubated with Alexa-488-conjugated goat anti-rabbit IgG for 3 h (Molecular Probes; 1:100 in 0.02 M PBS). The labeled tissues were mounted as cochlear surface preparations or slices of DRG in VectaShield (Vector Labs) and observed under a combined microscopy system (a Nikon Eclipse TE 300 inverted microscope fitted with a Bio-Rad MRC 1024 confocal scanning laser system). Mounted tissues were imaged using confocal optical slicing techniques, with a typical step size of 2 μm, and stacks of images were postprocessed for image analysis. For negative immunocytochemical controls, the primary antibody was replaced with either 1% BSA-PBS or primary antibodies adsorbed with a peptide encoding the predicted carboxyl terminus of VR1-EDAEVFKDSMVPGEK (Tominaga et al. 1998), prior to imaging at the same laser and confocal settings as experimental specimens.

Statistical analysis

Animals were divided into two major groups: the cochlear physiology group and the immunohistochemistry group. In the cochlear physiological group 36 guinea pigs were used. This group was further divided into four subgroups according to different measurements of the cochlear physiology and the use of different VR agonists: capsaicin and capsazepine on the CAP, CM, and EEOAEs ($n = 15$); RTX and capsazepine on the CAP, CM, and EEOAEs ($n = 10$); capsaicin and capsazepine on the CBF and EP ($n = 8$); and capsaicin and capsazepine on the BM ($n = 3$). However, the numbers shown in the results may be different due to different combinations of physiological measurement and chemical administration in each subgroup. Group data of VR agonists or antagonist-induced changes in cochlear physiology indexes are presented in means ± SE. An ANOVA with a repeated-measures design was utilized to determine significant difference across treatment groups. A probability of <0.05 is considered a statistically significant difference.

RESULTS

Effects of capsaicin on cochlear potentials and EEOAEs

The CAP, CM, and EEOAEs provide information on various aspects of cochlear function and therefore were used in this study as indexes of cochlear performance. It was found that perfusion of 20 μM capsaicin into the scala tympani resulted in reduction of cochlear sensitivity as shown by the alterations of the CAP and CM (Fig. 1). The CAP thresholds were tested with tones from 2 to 36 kHz that cover most of the hearing frequency range of guinea pigs. Elevation of CAP threshold by ~ 10 dB was observed after 20 μM capsaicin perfusion (Fig. 1*A*). To further investigate the effect of capsaicin on the auditory nerve activity, CAP response versus sound level (input-output) functions at 8 and 18 kHz were analyzed. The magnitude of the CAP was reduced by capsaicin by a small but statistically significant degree and with a parallel shift of the input-output function curves (Fig. 1, *B* and *C*). Furthermore, the reduction of the CM magnitude (at 8 and 18 kHz) by capsaicin was similar to that of CAP input-output functions in both reduction degree and pattern (Fig. 1, *D* and *E*). Both the CAP and CM recovered to normal after washout with artificial perilymph, indicating the effect of capsaicin administered in this experiment to be reversible (data not shown). In vitro experiments have shown capsaicin's effective concentrations for specific effects to be below 1 μM (EC_{50} = 0.52–0.9 μM) (Caterina et al. 1997; Welch et al. 2000). However, in our pilot experiment, we did not observe any effects of capsaicin until the concentration reached 10 μM, and the effects at this concentration were very subtle. At 20 μM, consistent and obvious effects on cochlear potentials were observed. Due to the concern of possible nonspecific effects at high concentration, we limited this study to 20 μM capsaicin. To verify the specificity of capsaicin's effects on the evoked cochlear potentials, capsazepine, a competitive antagonist of VRs which alone has no effect on cochlear potentials, was perfused into the cochlea in advance of capsaicin application. Capsaicin perfusion immediately after 20 μM capsazepine application did not affect cochlear potentials (Fig. 1). After capsazepine was washed out from the cochlea by artificial perilymph perfusion for ≥30 min, suppression of cochlear potentials by capsaicin application could be observed again. This indicates that capsazepine causes a complete and specific block of capsaicin's effects.

Capsaicin perfusion at 20 μM reduced overall EEOAE magnitude by ~5 dB without apparent effect on the fine structure (the peaks and notches in the magnitude spectra of EEOAEs; Fig. 2*A*). In certain cases (data not shown here), the notches in the fine structure became deeper, making the fine structure more profound. The effect of capsaicin on EEOAEs was reversible after washout with artificial perilymph (Fig. 2*B*). Also, perfusion with 20 μM capsazepine in advance blocked capsaicin's effect on EEOAEs (Fig. *C*). MCA showed that capsaicin reduced both the short and long delay components of the EEOAEs (Fig. 2*D*).

Effects of RTX on cochlear potentials and EEOAEs

To further verify that the observed capsaicin effects were mediated by VRs, effects on cochlear sensitivity by a potent agonist of VRs, RTX, was investigated. RTX has been shown to be at least ten times more potent than capsaicin (Caterina et al. 1997). Intracochlear perfusion of 2 μM RTX also reduced cochlear potentials and EEOAEs in a same manner as that induced by 20 μM capsaicin (see Figs. 3 and 4), suggesting the similar effects of capsaicin and RTX on cochlear sensitivity with ≥10 times difference in potency. However, recovery after washout of RTX was difficult to achieve. The effects of RTX could be completely blocked by capsazepine (20 μM) applied in advance.

Time dependence of CM alteration during capsaicin and RTX perfusion

To investigate the time course of hearing suppression effects by capsaicin and RTX, we monitored the alteration of the CM evoked by 8-kHz tone burst at 40 dB SPL during the VR agonist perfusion. Reduction of CM magnitude was observed within 1–2 min after capsaicin perfusion (Fig. 5, *A* and *B*). This effect was absent when capsazepine was applied in advance (Fig. 5*C*). In most animals ($n = 10$), the CM decreased gradually during perfusion (Fig. 5*A*). In some animals ($n = 2$), the CM decreased with a fast early reduction phase followed by a slower reduction phase (Fig. 5*B*). After the termination of capsaicin perfusion the CM showed a gradual recovery (Fig. 5*A*). The time course for the onset of RTX's effects was similar to that of capsaicin; however, we only observed the slow CM reduction pattern by RTX perfusion ($n = 5$). In the case of prolonged perfusion (≤20 min), a partial recovery in CM magnitude occurred ~15 min after the onset of perfusion with either capsaicin or RTX ($n = 7$ and $n = 5$, respectively; Fig. 5, *D* and *E*). The recovery of CM in this case is evidence of the desensitization phenomenon of VRs that has been observed in primary sensory neurons (Szallasi and Blumberg 1999).

Effect of capsaicin and RTX on cochlear blood flow and EP

Round window membrane application of capsaicin has been shown to increase cochlear blood flow (CBF) (Vass et at. 1995). In the current study, we found that intracochlear perfusion of capsaicin (20 μM) could increase CBF in a pattern similar to that with round window application (Fig. 6*A*). During a 10-min capsaicin perfusion, the CBF increased very quickly and reached the peak (about 140% of the initial level) in ~2 min. It fell to a level ~ 10% above the preexposure level in ~4 min and then had a slower return to the preexposure level. This CBF increase effect could be blocked by capsazepine.

Although an increase of CBF was observed, there is a possibility that the alteration of cochlear sensitivity might be the consequence of EP reduction. To test this possibility, the effect of capsaicin and RTX on EP was investigated in 8 animals. The EP was not altered with either capsaicin or RTX perfusion. Figure 6*B* presents the EP data during 20 μM capsaicin perfusion.

Effect of capsaicin on BM motion

BM velocity was measured at the 17-kHz best-frequency location in three animals. Data from these animals showed consistent alteration in BM motion by capsaicin. Figure 7 illustrates an example of such alteration in which the velocity growth functions near the best frequency (BF) were altered (Fig. 7*A*) and relatively unchanged at frequencies well below BF (Fig. 7*B*). These changes are typical for an insensitive cochlea, indicating a suppressed cochlear amplifier. The mechanical amplification of proposed cochlear amplifier is frequency dependent and most efficient at low sound levels. Thus it is the low sound-level portions of the growth functions near BF that are most affected. Figure 7*C* shows the iso-velocity tuning curve derived by determining the sound level required to produce a 10-μm/s criterion velocity response at various frequencies. The reduction of the cochlear sensitivity displays as a less sharply tuned curve. This is another hallmark of a reduction of the gain of the cochlear amplifier. Of particular note is that no change in the mechanical response of the BM occurred at low frequencies (e.g., 8 kHz). Therefore the lack of BM motion reduction by capsaicin at low frequencies cannot account for the reduction of the CM (e.g., Figs. 1 and 3) at these low frequencies. Because EP is also not reduced, a depolarization of the OHCs by capsaicin is the likely mechanism.

Immunolabeling and distribution of TRPV1 receptors in the rodent cochlea

Immunofluorescence microscopy was used to determine the distribution of TRPV1 in the organ of Corti of rat and guinea pigs using an anti-rat TRPV1 antibody. Confocal microscopy of the organ of Corti was conducted perpendicular to the lumenal surface of the sensory epithelium, resulting in horizontal optical sections of the cochlear surface preparation. In the rat organ of Corti, TRPV1 labeling was evident in the OHCs (Fig. 8*A*) with comparatively weaker labeling in the IHCs (Fig. 8*C*). TRPV1 expression could not be detected in the stereocilia of hair cells. Positive labeling for TRPV1 was also found in some supporting cells and in particular in the inner and outer pillar cells, and in Hensen's cells (Fig. 8, *A*–*C*, respectively). The labeling in outer pillar cells was somewhat weaker than in OHCs (average intensity = 70.7 ± 14.1; and 85.7 ± 11.3, respectively; $n = 10$; $P < 0.01$), and both cell types were significantly more intensely labeled than IHCs (45.5 ± 4.0; $P < 0.001$). TRPV1 expression was also found in the spiral ganglion neurons of the rat cochlea (Fig. 8*D*), confirming the previous report of TRPV1 in cochlear afferent neurons (Balaban et al. 2003).

In control experiments, when the primary TRPV1 antibody was replaced with PBS-BSA, negligible nonspecific labeling could be observed (Fig. 8, *E* and *F*). When the primary antibody was replaced with antibodies preadsorbed with a synthetic blocking peptide sequence (Tominaga et al. 1998), prior to incubation with organ of Corti material, negligible nonspecific labeling was observed in the organ of Corti (Fig. 8, *G* and *H*). In positive control experiments, strong immunolabeling for TRPV1 was found in rat DRG neurons (Fig. 8*I*) that are known to express TRPV1 in previous studies (Caterina et al. 1997). When the primary TRPV1 antibody was replaced with PBS-BSA or adsorbed with a synthetic blocking peptide sequence prior to incubation with rat DRG tissues, negligible nonspecific labeling could be observed (Fig. 8, *J* and *K*).

In the guinea pig organ of Corti, TRPV1 labeling was also present in the OHCs and IHCs but not in the stereocilia. Positive labeling for TRPV1 was also present in the outer and inner pillar cells, in Hensen's cells, and in the spiral ganglion neurons (data not shown). In guinea pig control experiments, when the primary TRPV1 antibody was replaced with PBS-BSA or antibodies preadsorbed with a synthetic blocking peptide sequence (Tominaga et al. 1998), negligible nonspecific labeling was observed in the organ of Corti. In positive control experiments, strong immunolabeling for TRPV1 was found in guinea pig DRG neurons and absent when the primary TRPV1 antibody was replaced with PBS-BSA or adsorbed with a synthetic blocking peptide sequence (data not shown). Thus the anti-rat TRPV1 antibody we used in guinea pigs showed the same pattern of positive labeling (albeit weaker) as in the rat.

DISCUSSION

VR-mediated mechanism for decreased cochlear sensitivity

Capsaicin and its ultrapotent analog RTX specifically activate VRs when used in proper concentration and hence have long been used to characterize the VR-mediated physiology of sensory neurons (e.g., Helliwell et al. 1998; Szallasi and Blumberg 1999; Szallasi et al. 1999) and nonneruronal cells (e.g., Biro et al. 1998a,b). Also, it is known that capsaicin has nonspecific effects (non-VR-mediated capsaicin actions) on other ion channels as well as various enzymes and cell membrane properties when applied with high concentration (Bevan et al. 1992; Szallasi and Blumberg 1999). Nonspecific effects can be ruled out for three reasons in the current study. *1*) Although the capsaicin concentration we used for perilymphatic perfusion was ≥20 times higher than for in vitro experiments (EC_{50} = 0.52–0.9 μM) (Caterina et al. 1997; Welch et al. 2000), the effective concentration in the organ of Corti will be much less than that in the perilymph because of diffusion from the perilymph

of the scala tympani into the organ. *2*) The action of 20 μM capsaicin on the cochlear sensitivity was completely blocked by capsazepine, a competitive VR antagonist that is effective against both capsaicin and RTX (Bevan et al. 1992; Maggi et al. 1993; Perkins and Campbell 1992; Szallasi and Blumberg 1999; Urban and Dray 1991; Wardle et al. 1997). *3*) RTX was used at a concentration within its effective concentration range as determined by in vitro experiment (Docherty et al. 1997). Identical effects on cochlear sensitivity were seen for RTX, which has a unique spectrum of biological activities that is devoid of most undesirable, capsaicin-like side effects (Szallasi and Blumberg 1999). Therefore the findings in this study imply functional VRs in the cochlea. This possibility is reinforced by immunolabeling experiment showing the presence of TRPV1 receptor in the organ of Corti.

VRs are members of OSM-9 family in the TRP (transient receptor potential) channel superfamily of Ca^{2+}-permeable ion channels (Caterina et al. 1997; Harteneck et al. 2000; Minke and Cook 2002). Binding of capsaicin and other vanilloids to VRs initiates a complex and, as yet, poorly understood cascade of intracellular events, which not only lead to excitation of nerves but also desensitization and neurotoxicity (Caterina et al. 1997; Szallasi and Blumberg 1999). Electrophysiological studies have shown that the VR channel pore opening leads to cation (predominantly Ca^{2+}) influx and may in turn cause depolarization, resulting in neuron excitation (Bevan and Docherty 1993; Marsh et al. 1987; Szallasi and Blumberg 1999; Wood et al. 1988). In the current experiment, reduction of CM and EEOAEs suggests a primary site of capsaicin and RTX's action in the cochlea to be on the OHCs by the rationale given in the following text and to result in a suppressive effect. It is possible that the alteration of OHC function by vanilloids occurred through a VR-mediated downward-regulation of OHC motility. Arguments of this hypothesis will be further discussed in the following text.

Continuous monitoring of CM revealed the time course of capsaicin and RTX's effects on cochlear sensitivity being ~2 min after the onset of perfusion (Fig. 5). This may represent the time for these vanilloids to diffuse and access the OHCs in the organ of Corti and exert their effects. The CM magnitude gradually decreased during perfusion, reaching the maximum effect in ~15 min, suggesting the pattern of vanilloid concentration increase during the perfusion. It may also represent the time course for maximum activation of vanilloids at a given concentration. Longer continuous application of the vanilloids failed to sustain the effect at its maximum level (Fig. 5, *D* and *E*), indicating a possible desensitization phenomenon of vanilloids' action on the organ of Corti. It was speculated that desensitization is a consequence of agonist-induced conformational change in VR protein that decreases the responsiveness of the ion channel. (Liu and Simon 1996). The brief CBF increase effect by capsaicin may also be attributable to the desensitization. However, the time course and shape of this effect are different from that on the CM.

Characteristics of cochlear function modulation by vanilloids

Physiological results of this study suggest actions of vanilloids on at least two major sites in the cochlea. *1*) Primary sensory nerves on cochlear vessels. The capsaicin-induced increase of the CBF is identical to that reported by Vass et al. (1995). The targeted vessels may include vessels of the spiral modiolar artery (SMA) and its arterioles. The basis for a CBF change could be a capsaicin-stimulated release of SP from possible primary sensory fibers innervating the cochlea (Vass et al. 1995, 2001). Despite the alteration in CBF, the EP was not affected by either capsaicin or RTX, indicating the normal functioning of the stria vascularis that maintains the electrical and ionic environment of the endolymphatic compartment of the cochlea. The reduction of cochlear sensitivity by vanilloids therefore cannot be attributed to EP reduction. The most parsimonious explanation is as a consequence of the action of VR agonists on the organ of Corti, especially, on the OHCs. *2*) OHCs. Reduction of the CM by capsaicin and RTX suggests a primary effect on OHCs, as

these cells are the primary site for CM generation and their responses are known to dominate the CM signal measured at the round window (e.g., Patuzzi et al. 1989). Decrease of CM magnitude may represent the reduction in mechanical drive to the cell (i.e., less stereocilia motion) or reduction in transduction current passing into the cell from an altered electro-chemical potential (i.e., reduced EP or cell depolarization) or from an altered cell electrical conductance. The lack of change of BM velocity for low-frequency stimuli and the lack of EP reduction with capsaicin application indicate that the observed CM reduction is likely due to an OHC conductance increase from the TRPV1 channel that also depolarizes the OHC. However, we also observe TRPV1 immunolabeling in supporting cells of the organ of Corti, with pillar cells having the strongest signal. It is possible that altered mechanical properties of supporting cells could reduce the OHC stereocilia motion without apparent BM velocity change. Our data cannot rule out this possibility.

Further evidence that OHCs are affected by vanilliods is from the change in electro-mechanical transduction (as evidenced by EEOAE mean value change) and cochlear mechanics (BM motion). In our previous studies, we observed that fine structure of the EEOAEs was a feature of sensitive cochlea and was related to the long delay component of the EEOAEs, whereas the overall mean magnitude of EEOAEs was mainly related to the short delay component and was relatively less sensitive to OHC damage (Ren and Nuttall 2000; Zheng et al. 2001b). In addition, fine structure results from cancellation/ enhancement effects of multiple sound waves in the cochlea (Ren and Nuttall 2000). Preservation or enhancement of fine structure suggests that the relative strength of the multiple waves is maintained even in the face of decreased overall power of the OHC electromotile response. Multiple-component analysis of the EEOAE data in this study showed that both the long and short delay components were reduced. This observation provides evidence in favor of the above speculation.

Based on the physiological data, we propose a hypothesis for the vanilloid-induced suppression of OHC function. Activation of VRs on the OHCs (probably on the basolateral wall) by vanilloids may result in Ca^{2+} influx through the VR channels and may in turn cause OHC depolarization and intracellular Ca^{2+} release. Because the OHC membrane potential and the EP together provide electrical force for transduction current through the OHCs, reduction of the OHC membrane potential (i.e., OHC depolarization) in the face of unchanged EP will result in a decrease of the transduction current, and hence, a reduction of the AC receptor potential. Increase in conductance at the base of the OHCs (due to VR ionic channel opening) could also reduce the transduction current (by a “shunt”) and reduce the AC receptor potential produced by the stereocillia receptor current (Guinan 1996). Reduction in OHC receptor potential would consequently reduce the voltage-dependent OHC motion that is involved in the amplification of BM motion. In addition, Ca^{2+} influx-induced intracellular Ca^{2+} release and the subsequent intracellular events (probably through phosphorylation in proteins responsible for OHC motility) may play a role as well because elevated intracellular Ca^{2+} causes OHC contractions and stiffness increase in an ATP-dependent manner that reduces the OHC motility (Dallos et al. 1997; Holley 1996; Sziklai et al. 1997). The observed decrease in CM, EEOAE and BM responses from capsaicin and RTX thus are consistent with OHC depolarization and reduction in cochlear amplification.

Positive expression of TRPV1 receptors in the IHCs and in the spiral ganglion neurons of both rats and guinea pigs (also reported by Balaban et al. 2003) implies an action of vanilloids on these sites. Our electrophysiological and BM motion data are not sufficient to distinguish whether this is the case. However, it is interesting that a parallel shift of the CAP growth function is observed after capsaicin application (Fig. 1, *B* and *C*). Because BM velocity returns to control values at high sound levels (Fig. 7*A*), the residual effect on CAP could be from IHC or afferent neuron effects.

Potential roles of VRs in the cochlea

In primary sensory neurons VRs are activated by heat, protons, and mechanical force, serving to transmit nociceptive information and being responsible for neurogenic inflammation. The expression and functional roles of VRs in other tissues remains unclear, although Birder et al. (2001, 2002) find that bladder epithelial cells express TRPV1 and that TRPV1 knockout mice have altered bladder function, and Denda et al.(2001) and Inoue et al. (2002) find expression in epidermal keratinocytes. In the auditory system, we propose that the VRs may have roles in cochlear homeostasis and participate in pathophysiological process in the cochlea; however, the endogeonous agonists for VRs in the cochlea are not known.

First, there is a possible role of the VRs for cochlear nociception. Because the VRs are sensitive to pH and heat, alterations in pH and temperature could activate the VRs on the sensory fibers innervating the cochlear blood vessels. This would result in a brief increase of CBF and vascular permeability changes (Vass et al. 1995). The sensation that might be perceived from activation of such sensory afferents is not known. Second, there is a possible role of the VRs in regulating the activity of the organ of Corti. However, our data indicate that the effects of TRPV1 activation on the hearing sensitivity will be small. The sensory, supporting cells and spiral ganglion cells of the cochlea may be able to respond to environmental alterations in pH, temperature, and osmotic pressure. Protons (or reduction of pH) are able to activate TRPV1 in normal physiological temperature (i.e., 37°C) (Tominaga et al. 1998). Although cochlear temperature would be expected to be regulated tightly and the TRPV1 receptor is gated on at ~43 °C; however, it is also known that protons can sensitize the channel to a lower temperature activation threshold (Tominaga et al. 1998). Recently, VR-related osmotically activated channels TRPV4 (also known as VR-OAC or OTRPC4) were reported to be present in the organ of Corti (Dai et al. 2002; Liedtke et al. 2000). Specific ion channel activation-induced Ca^{2+} influx (through these channels) and subsequent Ca^{2+} release from internal stores triggered by such Ca^{2+} influx has already been observed in VR and VR-OAC channels in other tissues (Caterina et al. 1997; Liedtke et al. 2000). TRPV4 is also gated by temperature in the normal physiological range (Guler et al. 2002). The molecular relationship between VRs and their TRP family members makes it likely that this group of ion channels subserves diverse but related physiological functions. In addition, suppression in EEOAEs and BM response suggest that VRs may play a role in modulating the mechanics of the organ of Corti, presumably by changing the cell turgor or stiffness. Third is the possible role of VRs in cochlear pathophysiology. In the case of inflammation, a combination of endogenous vanilloids and inflammatory mediators may act on VRs, which in turn initiates the inflammatory cascade (Szallasi and Blumberg 1999). It is possible that the VRs may also play a similar role for hearing loss in inflammatory processes in the inner ear or in pathological situations that may affect inner ear lymph chemistry. Meniere's disease and autoimmune sensorineural hearing loss are examples of such conditions.

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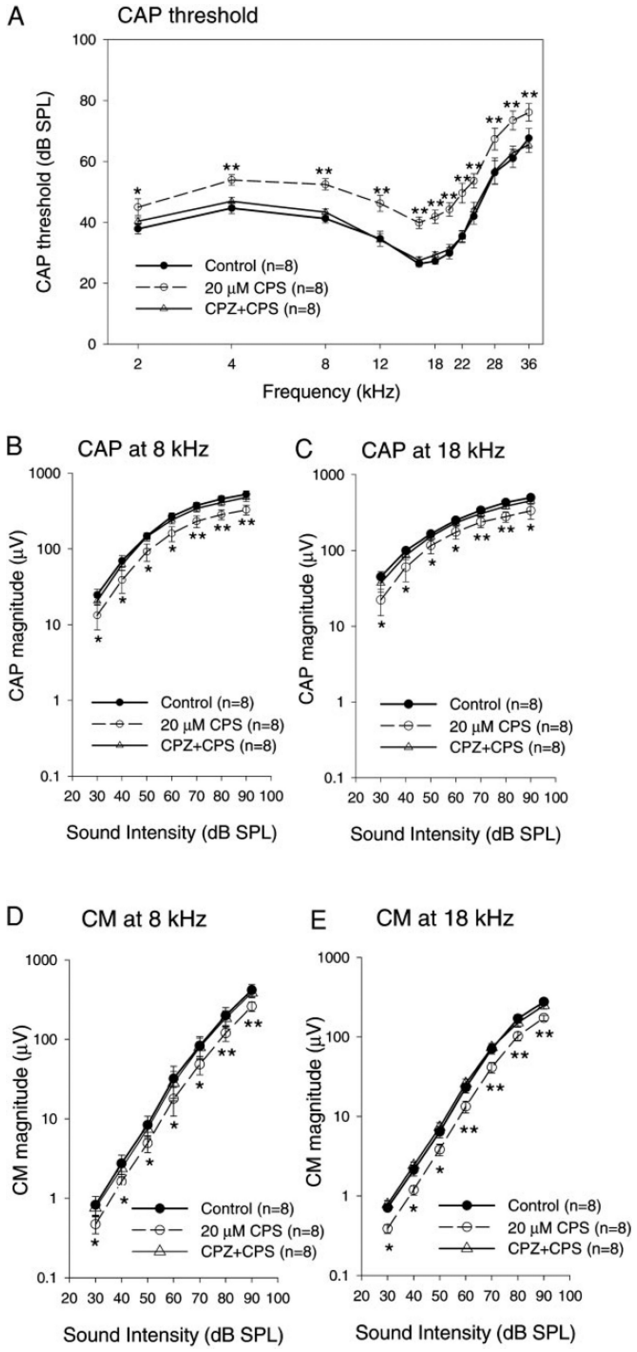

FIG. 1.

Effects of capsaicin (CPS) on cochlear potentials. Capsaicin (20 μM) and capsazepine (20 μM) were infused into the scala tympani of guinea pig cochlea. *A*: compoud action potential (CAP) thresholds from 2 to 36 kHz were significantly elevated by capsaicin (*, $P < 0.05$; **, $P < 0.01$). The effect of capsaicin could be blocked by capsazepine (CPZ), showing no significant threshold shift (see the CPZ+CPS) compared with control. *B* and *C*: CAP (8 and 18 kHz) input-output function curves shifted downward by capsaicin with statistical significance (*). *D* and *E*: cochlear microphonic (CM; 8 and 18 kHz) input-output function curves shifted downward by capsaicin with statistical significance (*). The effect of capsaicin on CAP and CM input-output functions could be blocked by capsazepine (see CPZ

+CPS). Vertical bars represent SE. *, statistical significance in comparison with control groups (*$P < 0.05$; **$P < 0.01$).

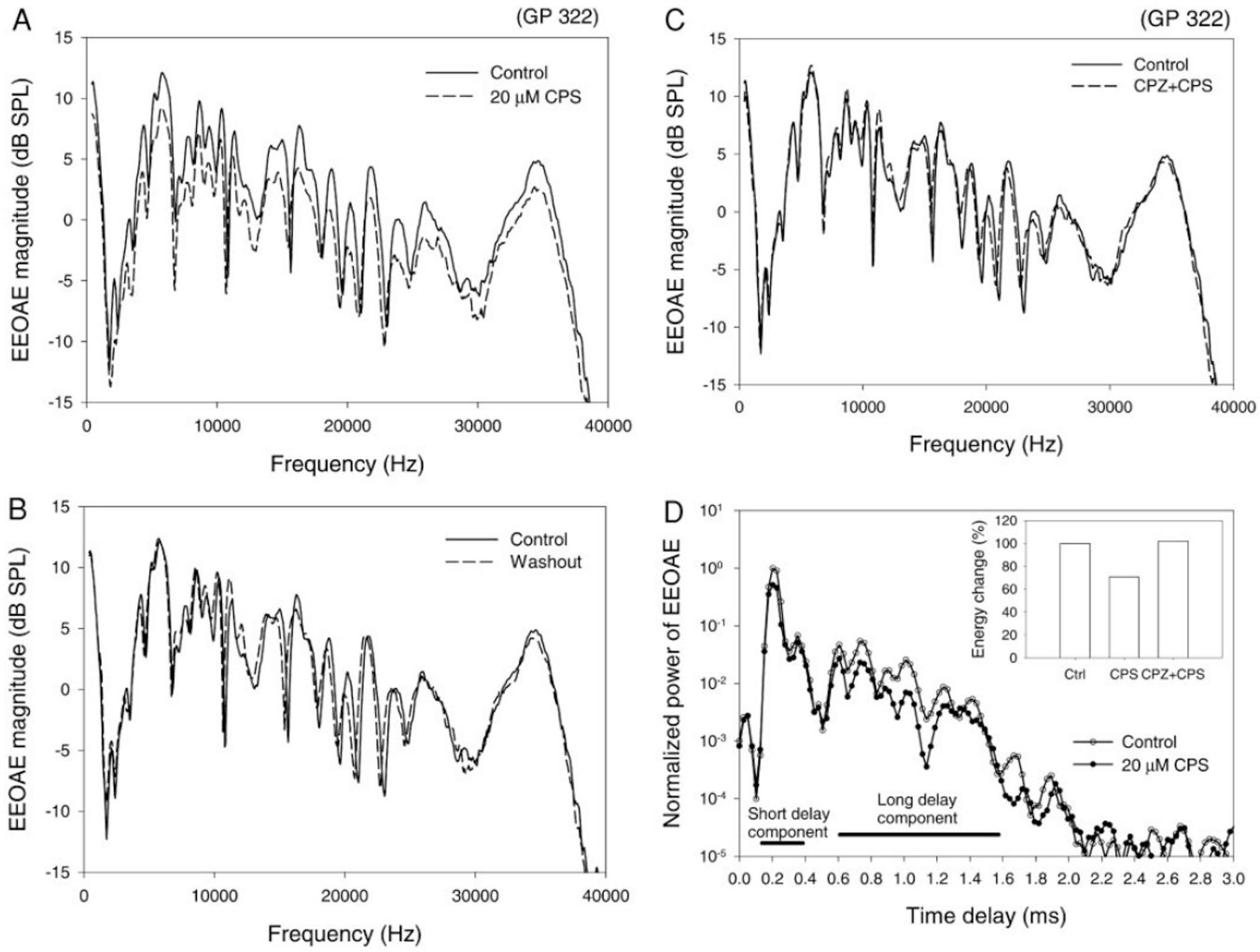

FIG. 2.

Capsaicin-induced electrically evoked otoacoustic emission (EEOAE) changes. The EEOAE was evoked by 35 μA RMS current injection to the round window. *A*: magnitude spectra of EEOAE with artificial perilymph (control) and 20 μM CPS perfusion. The small notches and peaks in the magnitude spectra are described as "fine structure" which is a feature of sensitive cochlea. CPS caused overall EEOAE magnitude reduction by ~5 dB while the fine structure showed no evident alteration. *B*: magnitude spectrum of EEOAE with artificial perilymph washout after CPS administration. The EEOAE recovered to preexposure level. *C*: magnitude spectrum of EEOAE with 20 μM capsazepine (CPZ) perfusion followed by CPS application (CPZ+CPS). The effect of capsaicin was blocked by capsazepine so that the EEOAE magnitude and fine structure appeared almost the same as the control. *D*: power vs. delay of the EEOAE by multiple component analysis. The time windows for short and long delay components are indicated by horizontal bars at the bottom of the figure. Both the short and long delay components were affected by capsaicin (i.e., reduction in power). *Inset*: the percentage of energy alterations with capsaicin and capsazepine perfusion.

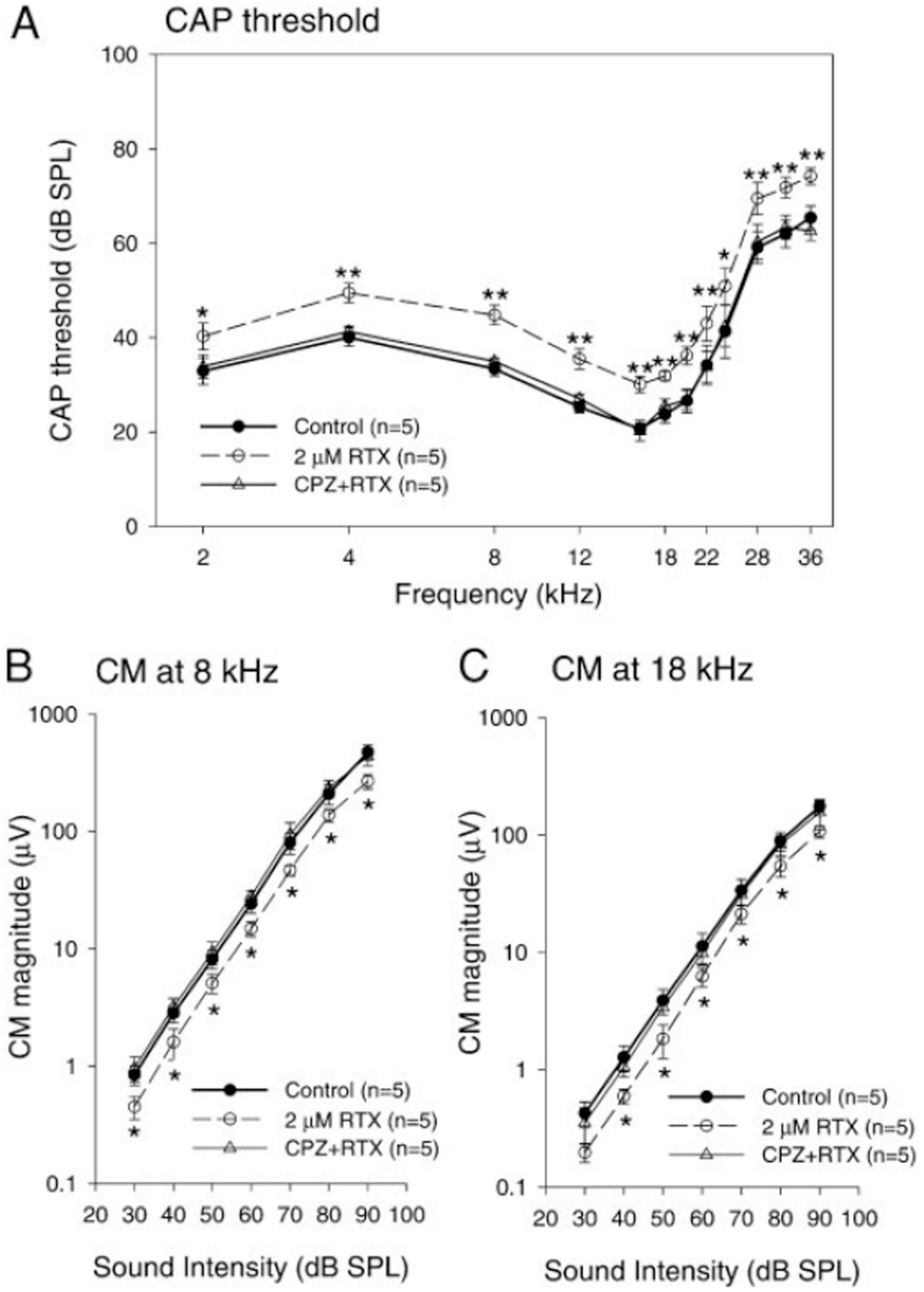

FIG. 3.

Effects of 2 μM resiniferatoxin (RTX) on cochlear potentials. *A*: CAP threshold. *B* and *C*: CM magnitude input-output function at 8 and 18 kHz, respectively. The CAP threshold was significantly elevated and the CM magnitude was decreased by RTX, which was similar to that by capsaicin. The effect of RTX could be blocked by 20 μM CPZ, showing no significant shift in CAP threshold nor CM magnitude input-output function curves (see the CPZ+RTX) compared with control. Vertical bars represent SE. **P*< 0.05; ***P*< 0.01.

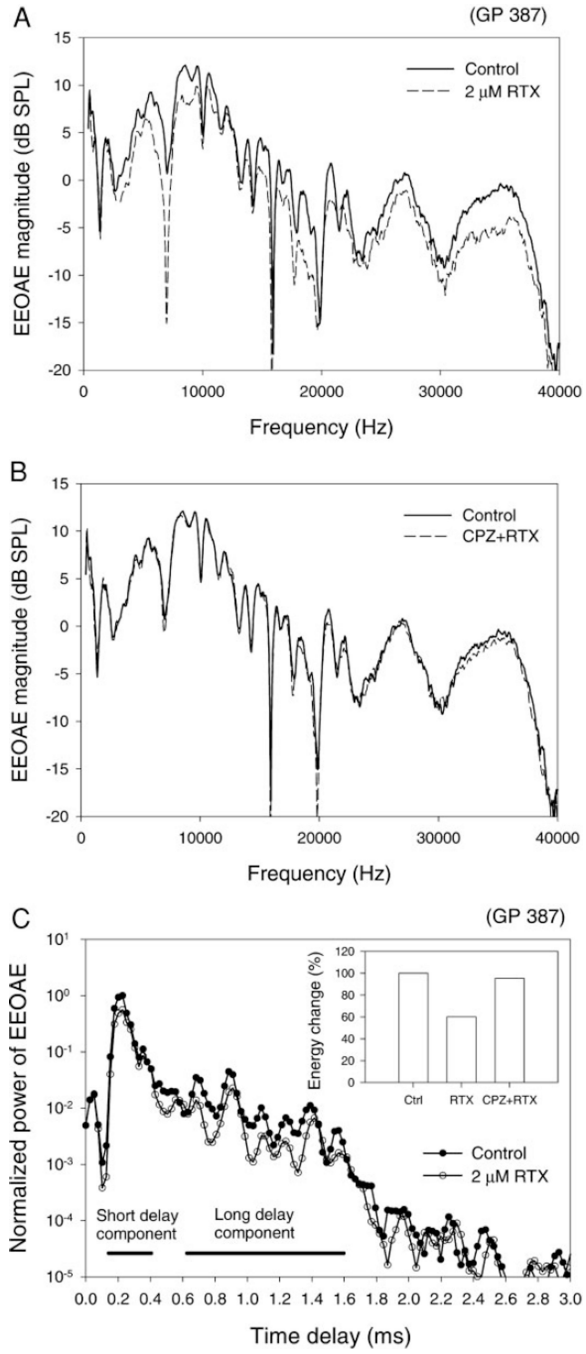

FIG. 4.

RTX-induced EEOAE changes. *A*: magnitude spectra of EEOAE with artificial perilymph (control) and 2 μM RTX perfusion. The RTX reduced the overall magnitude of EEOAEs without a diminution effect on the fine structure, showing a similar effect to that of capsaicin. *B*: magnitude spectrum of EEOAE with 20 μM CPZ perfusion followed by RTX application. The effect of RTX was blocked by CPZ as the EEOAE magnitude spectrum after CPZ+RTX perfusion overlaps that of the control. *C*: power vs. delay of the EEOAE by multiple component analysis. The time windows for short and long delay components are indicated by horizontal bars at the bottom of the figure. Both the short and long delay

components were affected by RTX (i.e., reduction in power). *Inset*: the percentage of energy alterations with RTX and CPZ perfusion.

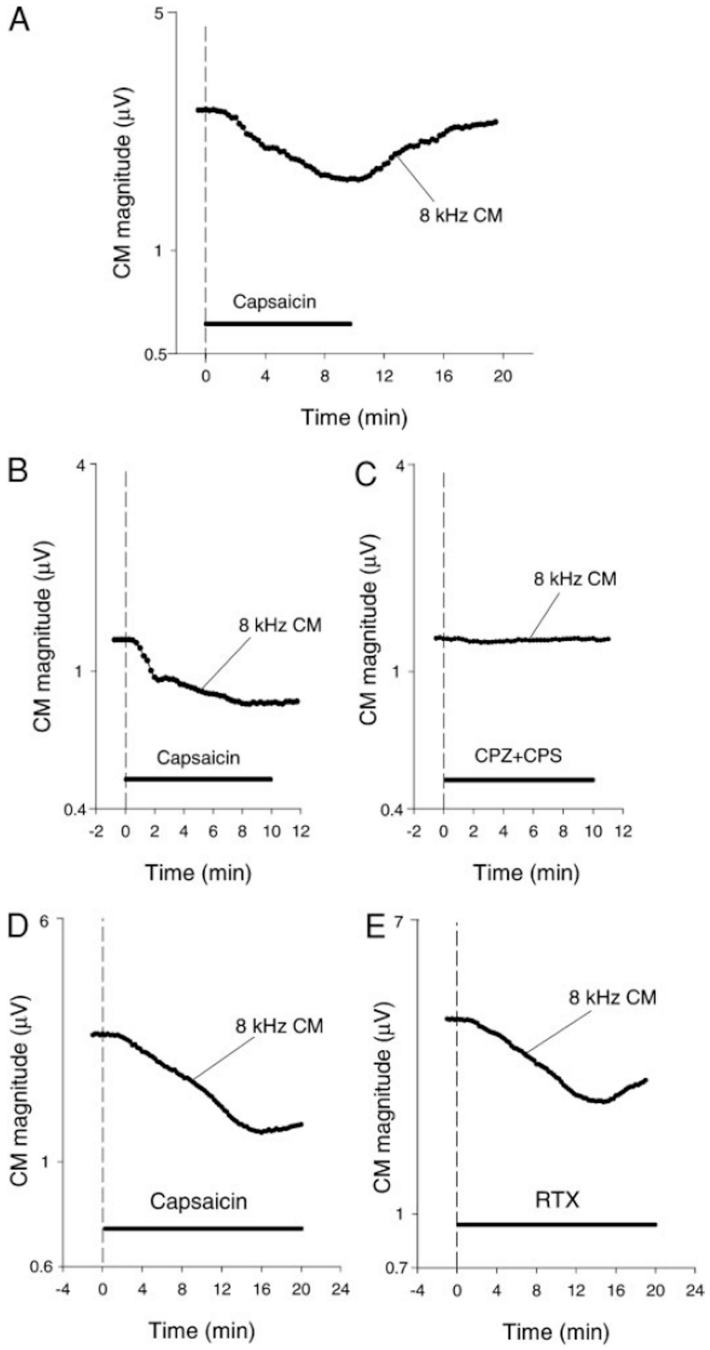

FIG. 5.

CM (8 kHz) magnitude alterations during 20 μM capsaicin or 2 μM RTX perfusion. *A*: CM decreased gradually during a 10-min perfusion with 20 μM CPS. After the termination of perfusion, the reduction of CM could last for ≥10 min with a gradual recovery. *B*: CM decreased with a fast and then slow phases during 20 μM CPS perfusion. *C*: CPS perfusion had no effect on CM magnitude when CPZ was applied in advance. *D* and *E*: CM (8 kHz) magnitude alterations during prolonged perfusion with 20 μM CPS or 2 μM RTX. It is noticed that CM magnitude reduction by both CPS and RTX reached its lowest level at ~ 15 min. Then the CM showed slight return even though the perfusion continued. The vertical

dashed lines indicate the onset of perfusion. Horizontal bars at the bottom of each figure indicate the duration of perfusion.

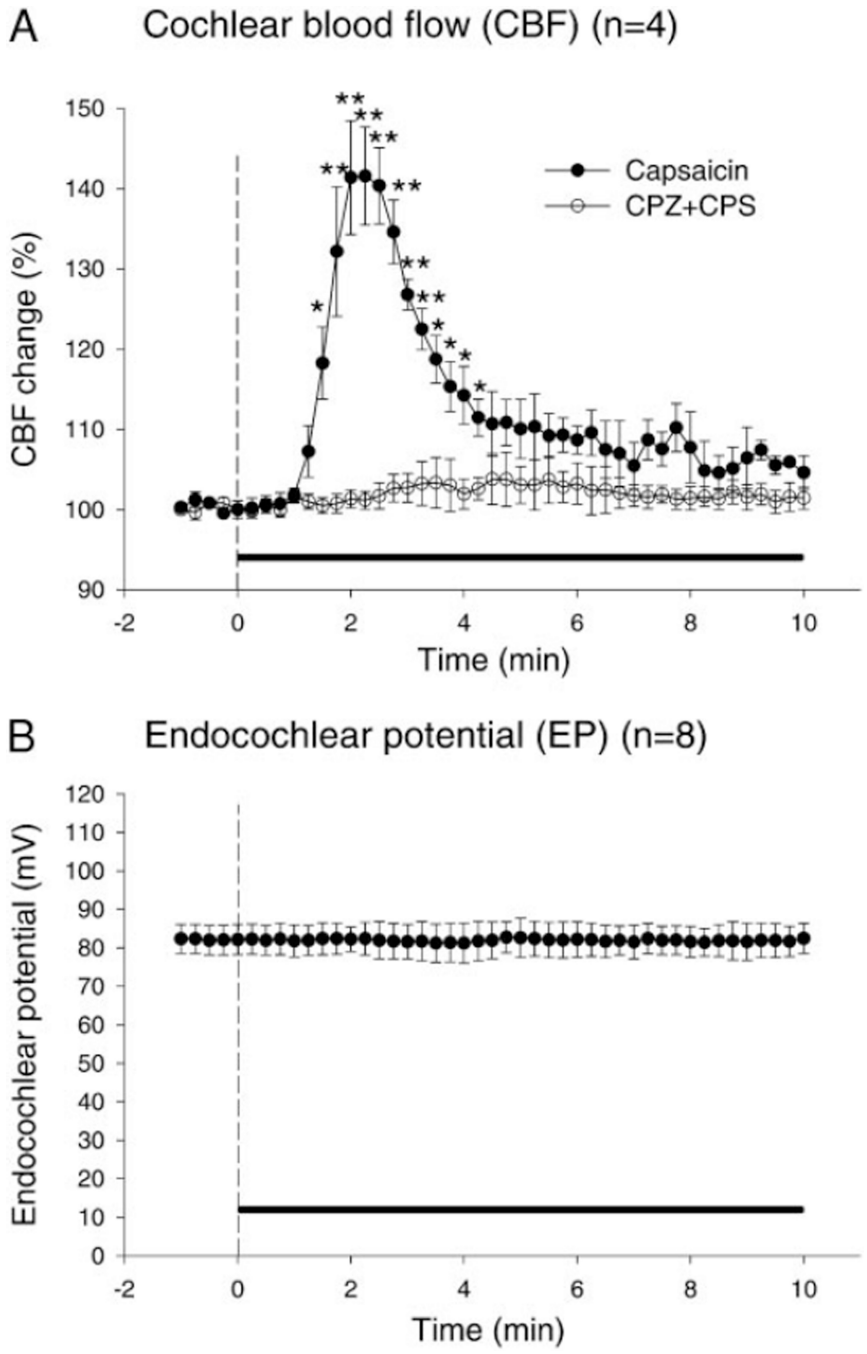

FIG. 6.

Cochlear blood flow (CBF) and endocochlear potential (EP) during capsaicin perfusion. *A*: CPS-induced CBF change. The change of CBF is presented as percentage value relative to preexposure level. The CBF increased quickly after 20 μM CPS perfusion and then fell down in a few minutes to a level just slightly higher than the preexposure level. When CPZ was applied in advance, CPS did not induce CBF increase (− O −). *B*: EP was not altered by CPS perfusion. Vertical bars on the curves represent SE. Horizontal bars at the bottom of each figure indicate the duration of CPS perfusion. $*P < 0.05$; $**P < 0.01$.

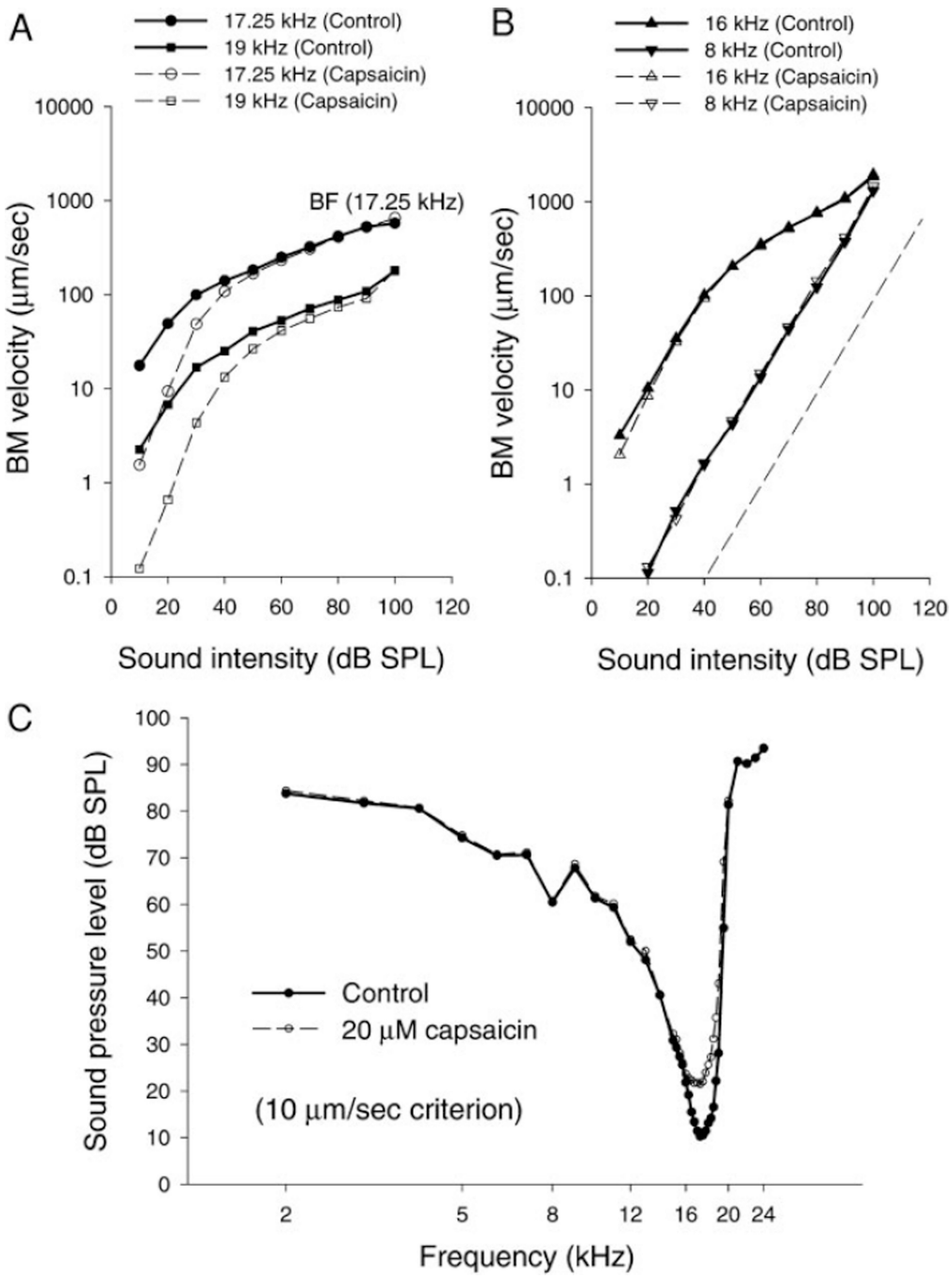

FIG. 7.

Basilar membrane (BM) velocity responses and iso-velocity tuning curve. *A* and *B*: BM velocity growth functions (input/output curves) for 4 representative frequencies (of 36). The curves for near best frequency (BF) (at 17.25 kHz) exhibit compressive nonlinear growth patterns that are altered by capsaicin for low sound levels. Frequencies much lower than BF have linear growth and are not affected by CPS (e.g., 8 kHz in *B*). - - - in *B*, the slope of linear growth. *C*: the frequency tuning curve derived using a 10-μm/s criterion. The tip of the tuning curve is vulnerable to capsaicin application.

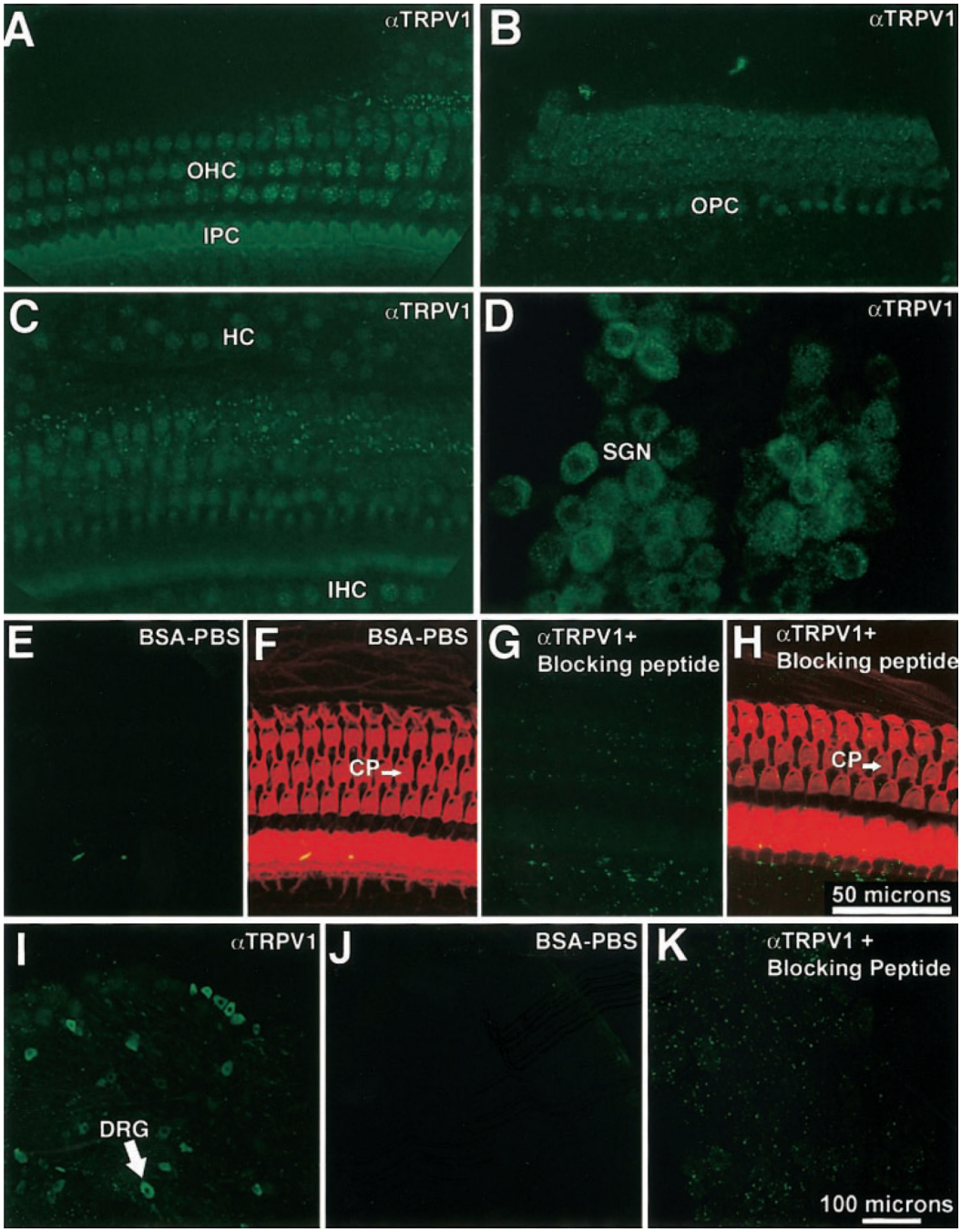

FIG. 8.

Immunolabeling of TRPV1 in the rat cochlea and dorsal root ganglion (DRG). *A*: TRPV1 immunolabeling was present in the soma of outer hair cells (OHCs), and inner pillar cells (IPC). *B*: TRPV1 expression was also identified in the soma of outer pillar cells (OPC), and in *C*, in Hensen's cells (HC) and IHCs. *D*: spiral ganglion neurons (SGN) also expressed TRPV1 labeling. *E*: immunocytochemical control: negligible labeling was observed in the rat organ of Corti when the TRPV1 primary antibody was replaced by BSA-PBS. *F*: phalloidin labeling (red) of the organ of Corti shown in *E*, showing the actiniferous cuticular plate of OHCs (CP, arrow). *G*: Immunocytochemical control: negligible labeling in the rat organ of Corti was observed when blocking peptide was added to TRPV1 primary antibody

incubation media. *H*: phalloidin labeling of the organ of Corti shown in *G*. The bar in *H* represents 50 μm and applies to *A–H*. *I*: specific TRPV1 immunolabeling occurs in the cell body of small-diameter DRG neurons. *J*: immunocytochemical control: negligible labeling in the DRG was observed when the TRPV1 primary antibody was replaced by BSA-PBS. *K*: immunocytochemical control: negligible labeling in the DRG was observed when blocking peptide was added to TRPV1 primary antibody incubation media. The bar in *K* represents 100 μm and applies to *I–K*.