CONTRIBUTION OF BK Ca\textsuperscript{2+}-ACTIVATED K\textsuperscript{+}-CHANNELS TO AUDITORY NEUROTRANSMISSION IN THE GUINEA PIG COCHLEA

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ABSTRACT

Large conductance calcium-activated potassium (BK) channels are known to play a prominent role in the hair cell function of lower vertebrates where these channels determine electrical tuning and regulation of neurotransmitter release. Very little is known, by contrast, about the role of BK channels in the mammalian cochlea. In the current study, we perfused specific toxins in the guinea pig cochlea to characterise the role of BK channels in cochlear neurotransmission. Intracochlear perfusion of charybdotoxin (ChTX) or iberiotoxin (IbTX) reversibly reduced the compound action potential (CAP) of the auditory nerve within minutes. The cochlear microphonics (CM at f1= 8kHz and f2=9.68kHz) and their distortion product (DPCM at 2f1-f2) were essentially not affected, suggesting that the BK specific toxins do not alter the active cochlear amplification at the outer hair cells (OHCs). We also tested the effects of these toxins on the whole-cell voltage-dependent membrane current of isolated guinea pig inner hair cells (IHCs). ChTX and IbTX reversibly reduced a fast outward current (activating above -40 mV, peaking at 0 mV with a mean activation time constant $\tau$ ranging between 0.5 to 1 ms). A similar block of a fast outward current was also observed with the extracellular application of barium ions which we believe permeate through Ca$^{2+}$ channels and block BK channels. In situ hybridization of Slo antisense riboprobes and immunocytochemistry demonstrated a strong expression of BK channels in IHCs and spiral ganglion, and to a lesser extent in OHCs. Overall, our results clearly revealed the importance of BK channels in mammalian cochlear neurotransmission and demonstrated that at the pre-synaptic level, fast BK channels are a significant component of the repolarizing current of IHCs.
INTRODUCTION

Ca\(^{2+}\)-activated K\(^+\) channels have been described in most excitable cells and have been classified into three subfamilies according to their single-channel conductance, calcium sensitivity, voltage dependence and pharmacology (for review see Hinrichsen 1993; Vergara et al. 1998).

Small conductance Ca\(^{2+}\)-activated K\(^+\) channels (SK channels) have a small unitary conductance (<20 pS), are generally voltage-independent, sensitive to the bee venom toxin apamin, and are activated by an increase in the levels of intracellular Ca\(^{2+}\) such as occurs during an action potential. SK channels generally underlie the slow afterhyperpolarization that limits firing frequency during a train of action potentials (see Sah 1996). Three mammalian SK channels (SK1, SK2, SK3) that demonstrate a high degree of homology and high sensitivity to Ca\(^{2+}\) have been cloned by Köhler et al. (1996). SK channels, and in particular SK2 channels, are expressed in cochlear outer hair cells (OHCs) (Dulon et al., 1998; Oliver et al., 2000). These SK channels underlie the fast cholinergic hyperpolarization of cochlear OHCs. They are functionally coupled to Ca\(^{2+}\)-permeable nicotinic receptors, composed of \(\alpha 9\) and \(\alpha 10\) nAChRs subunits (Elgoyhen et al., 1994; 2001), at the post-synaptic OHC membrane (Blanchet et al., 1996).

Intermediate Ca\(^{2+}\)-activated K\(^+\) channels exhibit unit conductance values of 20-80 pS, are voltage-independent and sensitive to apamin. The molecular basis of this family of potassium channels is poorly defined and they have not yet been described in the mammalian cochlea.

Large Ca\(^{2+}\)-activated K\(^+\) channels, named BK or maxi-K channels, display a high unitary conductance (ranging from 75 to 250 pS), are activated by both membrane depolarization and intracellular Ca\(^{2+}\), and are blocked by scorpion peptidyl toxins such as charybdotoxin (ChTX) and iberiotoxin (IbTX) (Miller et al., 1985; Galvez et al., 1990). BK
channels are generally hypothesized to accelerate action potential repolarization at synapses and terminate transmitter release (Patillo et al., 2001). These channels are composed of two structurely distinct subunits, α and β. The α subunit is a member of the Slo family of potassium channels (Knaus et al. 1994) and the gene encoding Slo was first isolated from Drosophila (dSlo) utilizing a genetic approach (Atkinson et al., 1991). Subsequent cloning experiments have revealed numerous splicing variants of Slo channels which produce various protein isoforms that differ in their sensitivity to calcium and voltage (Adelman et al., 1992).

BK channels are known to play a prominent role in hair cell function of lower vertebrates such as amphibians, reptiles and avians (for review see Fettiplace & Fuchs, 1999). These channels are colocalized with Ca\(^{2+}\) channels at sites of transmitter release, the hair cell's pre-synaptic active zones (Roberts et al., 1990; Issa & Hudspeth, 1994). BK channels are believed to regulate neurotransmitter release coupled to the calcium current at the hair cell's active zone as at the nerve-muscle synapse (Robitaille et al., 1993; Patillo et al., 2001). In electrically tuned hair cells, the number and the kinetic properties of BK channels (slo splice variants) determine the resonant frequency of each hair cell along the basilar papilla (Rosenblatt et al., 1997; Navaratnam et al., 1997; Jones et al., 1999; Ramanathan et al., 1999). Electrical resonance acts as an electrical filter maximizing the hair cell response at a specific sound frequency (Crawford & Fettiplace, 1981). Very little is known, by contrast, about the role of BK channels in mammalian cochlea neurotransmission. Although Ca\(^{2+}\)-activated K\(^{+}\) conductance has been described in both OHCs (Housley & Ashmore, 1992) and inner hair cells (IHC) (Dulon et al., 1995; Kros et al., 1998), there is still no direct evidence of Slo protein expression in mammalian cochlear hair cells. Mature cochlear IHCs, which constitute the pre-synaptic terminals connected to the auditory nerve fibres, do not display electrical resonance or electrical tuning. Based on earlier work (Johnson, 1980), Palmer & Russell (1986) showed phase-locking (indicative of rapid modulation of neurotransmitter release) up
to several kHz with mechanotransduction for these cells. A fast K$^+$ conductance (IK$f$), probably involving BK channels and first appearing at post-natal day 12 in mouse IHCs, is believed to transform mature mammalian IHCs into high-frequency signal transducers (Kros et al., 1998). The purpose of the current study was to characterize the role of BK channels in sound-evoked auditory nerve action potentials in vivo in the guinea pig cochlea, using the perfusion of specific toxins into the scala tympani. In addition, we studied the cellular pattern of expression of Slo channels in the mammalian inner ear with in situ hybridization of specific riboprobes and immunocytochemistry. The presence of fast BK channels was also characterized and confirmed at the pre-synaptic level on isolated adult guinea pig IHCs.
MATERIAL AND METHODS

Experimental animals and anesthesia

Experimental animals were female albino guinea pigs (GPs) (250-300 g) with a normal acoustic pinna reflex. The GPs were anaesthetised with intra-muscular injections of 1ml/kg of a mixture of two volumes of Ketamine Chlorhydrate (Ketalar, Parke Davis, 50 mg/ml) and one volume of 2% Xylazine (Rompun, Bayer). Such anaesthesia produced deep sedation of the animal while conserving spontaneous breathing.

Animal handling throughout these experiments was performed with authorisation of the French Ministry of Agriculture and in accordance with EEC regulations.

Intracochlear perfusion and sound-evoked potential recordings

After infiltration of local anaesthesia of 1% Lidocaïne (Astra), a tracheotomy was performed and a 2 cm polyethylene tube inserted and sutured in the trachea. The animal’s head was then secured dorsally in a stereotactic apparatus by a mouth-piece and hollow ear-bars. Body temperature was measured with a rectal probe and maintained with a heating blanket at 37°C (± 1°C). The bulla of the left ear was approached ventrally and its bony framework opened. Three holes were made into the cochlea: two in the scala vestibuli (SV) and one in the scala tympani (ST). A hole was initially made in the SV at the first turn of the cochlea for the recording electrode. Electrophysiological responses to acoustic stimuli were recorded versus the indifferent and ground needle electrodes in the neighboring tissues. A ST hole was then made in the basal turn of the cochlea for the perfusion catheter. A second SV hole was finally made into the first turn to release perilymph during perfusion to prevent
pressure build-up. Only when the recordings at this stage were not significantly different from that performed before the placement of the catheter was the experiment allowed to proceed.

The recording electrode was made of Teflon insulated stainless steel wire (125/175 µm bare/coated wire diameters) with a ½ mm uncoated end sealed to the Teflon sheath with a drop of cyanoacrylate glue serving both as a stop and seal against the hole in the cochlea wall. The perfusion catheter was made of a 5 mm stainless steel micro-tube of 0.127/0.229 mm inside/outside diameters (Gauge 34, Phymep, France) connected at one of its ends to a polyethylene catheter. The stainless steel micro-tube was introduced up to 4 mm inside the polyethylene catheter and glued with a drop of cyanoacrylate glue. The steel micro-tube end was introduced inside the cochlea through the hole in the scala tympani of the basal turn. The other end of the polyethylene catheter was connected to a 50 µl Hamilton syringe positioned in an electric syringe pump (Type 101, Phymep). The perfusion rate was set at 1.75 µl/min throughout the experiments.

Electrophysiological measurements were performed on the GP in a double wall sound proof room (IAC, New York, USA). The signal generating and recording equipment was situated in an adjacent room, except for a battery-operated preamplifier which was placed inside the sound proof room. In order to investigate the effects of the perfused drugs, cochlear microphonic (CM) and whole nerve compound action potentials (CAP) were measured on responses evoked by tone bursts presented in a Sennheiser speaker (HD 480 II, Germany), mounted in aluminum housing and connected to the left hollow ear bar by silastic tubing. The tone burst stimuli were generated using a PC-based waveform generator (Tucker and Davis Technologies, Gainsville, Florida, USA). The tone bursts had a duration of 20 ms, with 2 ms rise/fall times. The frequency used, taking into account the placement of the recording electrode and of the catheter along the cochlear partition (middle of first turn), was 8 kHz. The tone bursts were presented at levels of 30 to 90 dB SPL at a repetition rate of 12/sec. In
preliminary experiments, sound levels were calibrated with a Bruel-&-Kjaer 4134 microphone and a calibrated probe-tube placed inside the ear-bar, close to the tympanic membrane. Correct coupling of the ear-bar to the ear was visually controlled in each experiment by observing a slight tympanic membrane displacement when applying pressure at the other end of the ear-bar, before introduction of the silastic tube from the speaker assembly.

In some experiments, measurement of 2f1-f2 distortion products in the cochlear microphonics (DPCM) were made using two simultaneous tonebursts at 8 kHz (f1) and 9.68 kHz (f2) at levels from 30 to 80 dB SPL. These two sounds were presented in 2 separate Sennheiser speakers connected to the hollow left ear bar via a Y silastic tubing. Off-line, using a custom software developed on LabView (National Instruments), the recordings were automatically analyzed and measured. First, the electrical compound response recorded from the scala vestibuli (Fig. 1) was low-pass filtered (< 3 kHz; digital filtering with LabView) and CAP measured from baseline to first negative trough (N1) (Fig. 1B). Then the original response was high-pass filtered (> 3 kHz) and CM amplitude measured (Fig. 1B). In the experiments using two simultaneous tone bursts, CM amplitudes at f1, f2 and DPCM (2f1-f2) frequencies were measured on the amplitude frequency spectrum of the raw recorded response. All analysis and measures were made using digital signal processing through LabView software. The FFT analysis of the CM signal was so performed and the amplitude of the peaks at F1, F2 and 2F1-F2 automatically measured in dB re 1 µV RMS (Fig. 1C). It is worth noting that although the two sounds f1 and f2 were always kept at the same level SPL, the microphonic spectrogram displayed two primary components separated by about 20 dB. This was most likely due to changes in microphonic sensitivity at f1 and f2.
**Inner hair cell preparation and whole-cell patch clamp recordings**

Inner hair cells were isolated from adult GP cochleae as previously described (Dulon et al., 1995; Sugasawa et al., 1996). The two lower turns of the organ of Corti were removed from the bony shell of the cochlea and placed in Hanks' balanced salt solution (HBSS; Sigma), containing in mM: NaCl 136.9, KCl 5.4, MgSO$_4$ 0.81, CaCl$_2$ 1.26, KH$_2$PO$_4$ 0.44, Na$_2$HPO$_4$ 0.34, N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) 5, and glucose 5.5. The Hanks' solution was adjusted to pH 7.35 with 2mM NaOH and to 300 mosm/kg H$_2$O with 6 mM NaCl. The organ of Corti, freed from the bony cochlea, was then incubated for 10 minutes in a 40 µl drop of HBSS containing collagenase (type IV, final concentration 0.8-1 mg/ml, Sigma). The pieces of organ of Corti were transferred to a 50 µl drop of HBSS in the middle of a glass coverslip sealed on the perforated bottom of a petri-dish. The cells were then mechanically dissociated with gentle influx and efflux with a Gilson micropipette (Gilson, France), and left to settle for 30 min. The dish was then filled with 3-4 ml of HBSS. Inner hair cells were recorded under voltage clamp configuration with electrodes pulled from borosilicate glass capillaries (GC150TF-10 Clark Electromedical, England), on a Sachs-Flaming horizontal electrode puller (Sutter Instruments, USA). Recording electrodes were back-filled with the following internal solution containing in mM: KCl 158, MgCl$_2$ 2, EGTA 1.1, HEPES 5, KOH 3.5, pH 7.20. Patch-clamp recordings were performed as previously described in detail (Blanchet et al., 1996) by means of an Axopatch-1D amplifier (Axon Instruments, Foster City, CA). Axotape and pClamp software (Axon Instruments) were used for data collection and analysis. Voltage errors attributable to uncompensated series resistance and liquid junction potential were corrected during data analysis (Blanchet et al., 1996). All experiments were done at room temperature (20-22°C).

The test solutions, toxins (from Latoxan, France) and controls, were applied to isolated IHCs by a Picospritzer puffer system (Picospritzer II, General Valve, Fairfield, NJ). Pipettes
for the Picospritzer system were pulled in a similar fashion to the recording patch-clamp pipettes and were placed at about 20-40 µm from the cells. The pressure of the perfusion system was set low enough to avoid any mechanical disturbance of the cell during current recording.

**Cloning rSlo from rat cochlea**

In order to prepare RNA probes for in situ hybridization, we isolated and cloned the Slo cDNA from rat tissue because the complete sequence was not known in the guinea pig. Harlan Sprague-Dawley rats (day 7) were deeply anaesthetised with a rodent cocktail (Ketamine 50mg/kg, Rompun 5mg/kg, and Acepromazine 1mg/kg). After decapitation, the brain was removed and immediately frozen at -70 °C. Total RNA was isolated from frozen samples using TRIZOL Reagent (Life Technologies), and then reverse transcribed into cDNA using the Superscript Preamplification System (Life Technologies) from which PCR amplifications were performed with specific primers. Initially, rSlo transcripts were amplified from rat brain using a pair of oligonucleotides designed from mSlo sequence (L16912 Butler et al., 1993). The sense and antisense primers were respectively: 5'-CTTGACTCGAAGTGAAGCTGC-3' (nucleotides 2002-2222) and 5'-GGGTGAGGATATTGTCATTG-3' (nucleotides 2877-2896). These primers bracketed the hydrophobic segments S9 and S10 of mSlo at the 3' region. The PCR consisted of 35 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min 30 sec. The PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. The PCR products were ligated into the pGEM-T vector (Promega) and sequenced using an ABI 373A automated DNA sequencing system (ABI). Two splice variants, a short isoform (homologous to mSlo) and a long isoform (+ 86 bp compare to mSlo) were identified and cloned. Only the short isoform was expressed in the organ of Corti by RT-PCR and was consequently tested for
in situ in the rat inner ear. Our antisense RNA probe spans between the S9-S10 region of the KCNMA or mSlo cDNA, a rather well conserved region between splice variants. Our probe should theoretically recognize all KCNMA splice variants found in the rat cochlea (Langer et al. 2003).

In situ hybridization of Slo antisense riboprobes

Sprague-Dawley rats (2 months old) were used for in situ hybridization. Complete serial sections from 3 different animals were analyzed. Tissue preparation was done as previously described (Dulon et al., 1998). Briefly, rats were deeply anaesthetised with pentobarbital (70mg/kg) intraperitoneally and then perfused transcardially with warm saline followed by acid shift paraformaldehyde: a two-stage perfusion using equal volumes of 4% paraformaldehyde (pH 6.5) followed by 4% paraformaldehyde with 0.05% glutaraldehyde (pH 9.5). Cochleae were dissected from temporal bones and decalcified in 8% EDTA with 4% paraformaldehyde at 4°C. Frozen sections (20 µm) were cut and mounted on superfrost plus slides (Fisher Scientific), air dried, and stored at -20°C for later use.

The protocol for in situ hybridization has been previously described in detail elsewhere (Simmons et al., 1989). Briefly, tissue samples were permeabilized with 2 mg/ml proteinase K for 30 min at 37°C. Linearized rat cDNA templates for short isoforms of rSlo were used to synthesise 35S-labeled antisense riboprobes using appropriate RNA polymerase and 35S-UTP. Corresponding sense strand riboprobes were synthesised and used as control to each probe for nonspecific hybridisation. The probes were hybridized to the tissues and incubated at 56°C overnight. The sections were then treated with RNase A and high stringency washes (low salt and high temperatures). Preliminary evaluation of hybridization was obtained by opposition of slides to X-ray film for 48-72 hours. Afterwards, the slides were coated with Kodak NTP-2 liquid autoradiographic emulsion and exposed at 4°C for 2-4 weeks depending on the strength of the signal obtained on the film. The slides were developed
in Kodak D-19 (2.5 min at 14°C) and fixed in Kodak fixer. The sections were counterstained through the emulsion with bis-benzamide (0.001% for 2min), which preferentially labels cell nuclei but also stains cell membrane and residual proteins. The sections were examined by fluorescence microscopy to identify cochlear structures and under dark microscopy to evaluate the distribution of autoradiographic grains. Only sections that had been hybridized, dipped, exposed and developed in the same series were used for this analysis.

**Immunocytochemistry of BK<sub>Ca</sub> channels**

Immunolocalisation of Slo channels was carried out on surface preparations of organ of Corti dissected from adult guinea pig using a polyclonal antibody obtained from Alomone labs. This primary antibody, raised in rabbit against the C-terminal part (residues 1098-1196) of mouse Slo α subunit, was diluted at 1:100. Fixed cochleae were decalcified and the organ of Corti was dissected turn by turn. The stria vascularis and the Reisner’s membrane were removed. The different turns of the organ of Corti were then washed, and incubated in biotinylated anti-rabbit secondary (dilution 1:200; Sigma) for 2.5 hours. All incubations were performed by adding triton X-100 at 0.1%. The signal was amplified with an avidin-biotin-horseradish peroxidase procedure (Vector’s laboratory), and visualized using diaminobenzidine as the chromogen.

For cryostat sections, cochleae were put in a gradient of sucrose 10% (2 hours), 20% (2 hours) and 30% overnight. Cochleae were embedded in OCT medium and were frozen at –20°C. Selected slides were pre-incubated free floated for immunoreaction. They were rinsed, placed on subbed slides, dehydrated, cleared, and mounted for microscopic observation. Two control experiments to reveal any nonspecific labelling were carried out: 1) omission of the primary antibody from the procedure and 2) preadsorption of the antisera with the antigenic peptide provided by Alomone labs.
In conclusion, we used in the present study three different major procedures in order to obtain a complete picture of the role of BK channels in the mammalian auditory system. First, we performed *in vivo* cochlear recordings and intracochlear perfusion of specific toxins to characterize the role of BK channels in sound-evoked cochlear potentials. Second, we worked at the cellular level *in vitro* to examine the IHC currents and verify the presence of BK currents at the presynaptic level. Finally, we used in situ hybridization and immunocytochemistry to characterize the expression of mRNA and BK proteins in cochlear tissue.
RESULTS

Intracochlear perfusion of TTX

In order to test the stability of our preparation, we have first run a large number of sham experiments where artificial perilymph (HBSS) was perfused (n = 8). In our experience, CAP and CM were generally stable over time for more than 2 hours. In experiments with the toxins, all guinea pig were pre-perfused with HBSS for about 30 min to verify the stability of CAP and CM. We first tested our perfusion system with the fugu toxin, tetrodotoxin (TTX), a potent blocker of voltage-dependent Na\(^+\) channels. Intracochlear perfusion of TTX is well known to block auditory nerve activity (Konishi and Kelsey, 1968; Evans and Klinke 1982; Zhang et al., 1999). In our study, TTX was diluted in HBSS and was perfused in the cochlea for 5 to 15 min at a concentration of 1, 3 or 30 µM. One GPs was tested at each of these concentrations (the effects of TTX were essentially irreversible). For the GP tested at 3 µM TTX, a decrease in CAP from 46 µV to 17 µV was observed within 3.5 min of perfusion (Fig 2). The CAP waveform became broader and its latency increased. These changes were only partially reversible after 3 hours of rinsing with HBSS. On the contrary, CM amplitude, which is believed to essentially reflect the AC receptor current of hair cells, did not change during TTX perfusion (Fig. 2). In a similar manner to Fig 2, in the other two GPs tested at 1 and 30 µM of TTX, CAP was also rapidly and completely suppressed, while CM remained unaffected.

Intracochlear perfusion of ChTX and IbTX

We first studied the effects of intracochlear perfusion of the scorpion toxin charybdotoxin (ChTX), a toxin well known to block BK channels (Garcia et al. 1991). ChTX was perfused at concentrations of 5 µM (n = 1) and 2 µM (n = 2). At 2 µM, CAP amplitude decreased by 50-70 % within 10-15 min and recovered within 20 min after the end of the
ChTX perfusion (Fig. 3). In this GP that was tested with a second ChTX perfusion, there was again a rapid diminution in CAP but this time we observed an incomplete recovery, presumably due to a slower removal or washout of the toxin from its targets. At 5 µM, CAP decreased by 68 % (from 45 µV to 14.3 µV) after 3 min perfusion and to 0 µV after 6 min. After rinsing with HBSS, full recovery of CAP amplitude occurred about 30 min after the end of the 5 µM ChTX perfusion. This indicated a good washout and a reversible binding of the toxin on its sites of action. In all animals tested with ChTX, during the whole experiment CM amplitude fluctuated and decreased over time by less than 5 dB. The variations of CM during the toxin application were not significantly different when compared to the CM changes observed in untreated animals –1.2 ± 3.4 dB (n = 8).

We tested the effects of iberiotoxin (IbTX), another scorpion toxin which is known to specifically block BK channels, unlike ChTX which can also block certain other type of potassium channels such as Kv1.3 (Galvez et al. 1990). Results were obtained from 6 GPs with different IbTX concentrations: 1 µM (n = 4), 2 µM (n = 1) and one GP was perfused successively with 1, 2, 0.5 and 5 µM. Several perfusions of the same concentration of toxin were occasionally repeated on the same GP. Between each IbTX perfusion a continuous rinsing perfusion of HBSS was maintained. Figure 4 shows recordings and amplitude changes of CAP in one GP during repeated perfusions with HBSS and 1 µM IbTX. Similar effects to those obtained with ChTX are observed on CM and CAP during IbTX perfusions. The effects of IbTX were reversible and could be repeated during a second application (Fig. 4). Maximal reduction in CAP amplitude was observed within 25 min after starting the perfusion of IbTX. Recovery in CAP amplitude to pre-IbTX perfusion levels was complete within 45 min of rinsing with HBSS. In the 3 other GPs perfused with 1 µM IbTX, changes in CAP amplitude followed the same pattern with no change in CM except in one which showed a slight increase in CM amplitude (by about 4.5 dB; Fig.4). In summary, with 1 µM IbTX, the mean
maximum decrease in CAP amplitude was of 57.9 ± 23 %. This maximum CAP decrease occurred within 26.5 ± 5.7 min (n = 4) while CM was essentially unaffected (mean variation of -0.5 ± 3.5 dB). The variation of CM with IbTX was not significantly different when compared with variation of CM measured over time in IbTX-untreated GP (-1.2 ± 3.4 dB n = 8).

Successive perfusions with 4 different concentrations of IbTX (1, 2, 0.5, 5 µM) with HBSS perfusion in between were also carried out in one other GP (Fig. 5). In that case the tone burst at 8 kHz was presented at 4 different sound levels (90, 70, 50 and 30 dB SPL). At 0.5 µM IbTX, the reduction in CAP (15-20 µV) was similar at all sound intensities used (Fig. 5B). At 1, 2 and 5 µM IbTX the reduction in CAP increased largely with sound intensity. For 70 and 90 dB SPL, the dose/response curve of block by IbTX, obtained by fitting of the data with a sigmoidal Hill equation, gave an apparent IC50 of 0.4 and 1 µM IbTX, respectively (Fig 5C).

We have also tested in two other guinea pigs the effect of an intracochlear perfusion of apamin (5 µM). Apamin is a specific blocker of another family of Ca^{2+}-activated K^+ channels, the SK channels (Köhler et al. 1996). These channels, in particular SK2, are known to be highly expressed in OHCs but not in mature IHCs (Dulon et al. 1998). Intracochlear perfusion of apamin did not affect the evoked cochlear potentials (CAP, CM or DPCM) while a pre or post-perfusion of IbTX (1µM) largely reduced CAP in the same animals.

In order to determine whether the action of IbTX on CAP was arising from a block at the OHCs amplification function, we measured in four additional guinea pigs the effects of the toxin (1µM) on the inter-modulation distortion products (DPCM) of the cochlear microphonics (CM) in response to two-tone stimuli at 5 sound intensities (40, 50, 60, 70, 80 dB SPL) (Fig. 6). While CAP rapidly decreased at all sound intensities, the amplitude of CM at f1, f2 and DPCMs (2f1-f2 ) remained stable during the application of IbTX. Slight changes
on f1, f2 and DPCM were only seen 15 min after the drop in CAP. Overall, these results suggested that the diminution of CAP by IbTX was essentially arising from an action at the IHC, IHC/afferent synapse or ganglion cells (see discussion) rather than at the OHCs function. This assumption was reinforced by an even larger decrease of CAP at high sound intensities such as 80-90 dB SPL (Fig 5 and Fig 6). At this high sound level, CAP generation is believed to by-pass the OHC cochlear amplifier.

**Evidence of fast BK currents in IHCs**

We have also studied under whole-cell voltage clamp the effects of the same BK-specific toxins on the time and voltage-dependent conductances of isolated GP IHCs. Cells were held at -60 mV and current responses to depolarisation in 10-mV increments were recorded (Fig. 7A). All recorded IHCs displayed fast developing non-inactivating outward currents which started to activate above -40 mV (n = 30). The activation time course of this fast current could be well fitted by a single exponential with a time constant $\tau$ of $0.6 \pm 0.2$ ms at 0 mV and 20°C (range 0.2 to 0.9 ms). These kinetics are in good agreement with those reported at 20°C in a previous study by Kros and Crawford (1990). Extracellular application of IbTX during the voltage-step protocol suppressed, in all IHCs tested (n = 8), the fast component of outward currents (Fig 7B). The remaining IbTX-insensitive current displayed a kinetic of activation reduced by about one order of magnitude ($\tau = 5.7 \pm 2.2$ ms at 0 mV and 20°C; range 2.1 to 8.1 ms).

Several concentrations of IbTX were tested: 0.05, 0.1 and 1 µM. A concentration of IbTX as low as 50 nM showed a maximum block of the fast component, suggesting that the IC50 was in the nM range as expected for a specific action of the toxin on BK channels. The large difference (more than one order of magnitude) in concentration for maximal inhibition between *in vivo* and *in vitro* may simply reflect a lower access of the peptide toxin to the BK
channels from the perilymph during perfusion in vivo. Indeed, in vivo, the toxin would have to cross the basilar membrane cells and the surrounding supporting cells of the organ of Corti that may act as diffusion barriers to the toxins.

The block by IbTX on the fast IHC currents was completely reversible within minutes after rinsing with normal HBSS (not shown). The IBTX-sensitive current was visualized by subtracting currents before and in the presence of IbTX (Fig 7C). This current displayed rapid activation and time inactivation at large depolarized potentials. The fast IbTX-sensitive currents activated with a fast time constants (τ) ranging from about 1 ms to less than 0.5 ms as displayed in Fig 7C and Fig 7D insert. The current-voltage relationship of the IbTX-sensitive current showed outward currents starting to activate above -40 mV, with amplitude reaching a plateau at 0 mV and finally outward rectifying above +10 mV (Fig. 7D). The current showed time inactivation that became faster with depolarization at potentials above 0 mV. In consequence, the I-V curve displayed a N-shape that was less pronounced at the peak current (triangle symbol) than near the steady-state current (circle symbol). This faster inactivation of the BK current at larger depolarization could either be due a voltage-inactivation or due to a faster decrease of calcium near the BK channels if one considers that these channels are activated consecutively to a voltage-activated calcium entry or/and release.

A similar fast component of the outward currents was also reversibly suppressed by 0.1 µM ChTX (n = 5; data not shown) and with the extracellular application of 4 mM barium ions during the same voltage-step protocol (n = 3; not shown). On the contrary, extracellular application of apamin (2 µM), a potent blocker of SK channels (Köhler et al. 1996), did not affect the outward currents of IHCs (n = 3; data not shown).

Expression of rSlo
The search for the expression of Slo mRNA was made in the rat cochlea because, unlike guinea pig, the complete cDNA sequence of the gene Slo of mouse and rat is published in GenBank. In situ hybridization of adult rat inner ear sections using $^{35}$S-labeled antisense riboprobes of rSlo showed high level of expression in IHCs and a somewhat lower level of expression in OHCs all along the cochlea (Fig. 8A). A strong expression was also observed in spiral ganglion from the base to the apex of the cochlea. Some expression was also noticed in stria vascularis but at a much lower level. Strong expression of Slo mRNA was also detected in the vestibular sensory epithelium (Fig. 8B).

The expression of $\alpha$ subunits was confirmed at the protein level in the adult guinea pig organ of Corti by immunocytochemistry (Fig. 9A-B). On surface preparation, strong immunostaining was observed in IHCs and to a lesser extent in the three rows of outer hair cells. Similar staining was observed in three guinea pigs. Immunolabelling was stronger at the base of the cochlea for OHCs and equivalent in all turns for IHCs (Fig. 9C). Immunolabelling at the IHC level was confirmed by the identification of individual labelled whole IHCs at the edge of the surface preparation and in cryosections (Fig. 9D). No labelling was seen in the afferent fibers below IHCs suggesting that BK channels are mainly expressed presynaptically. Immunoreaction in cryo-sections of whole guinea pig cochlea, confirmed the results obtained with surface preparations (Fig 10). In the organ of Corti, IHCs were labelled, particularly at their basal synaptic pole where strongly labelled dots were observed (Fig 10 C-D). The soma of spiral ganglion neurons were also labelled while their fibers were unstained, again suggesting that the expression of BK channels at the IHC-synapse was essentially presynaptic. Deiters cells and OHCs also were immunoreactive but with somewhat less intensity than IHCs.
DISCUSSION

This is the first demonstration that intracochlear perfusion of IbTX, a toxin specific to BK channels, rapidly and reversibly blocks the sound-evoked compound action potential (CAP) of the auditory nerve. The absence of a concomitant large change in CM or DPCM suggests that the site of action of the toxin is essentially at the IHC-afferent nerve fibre synapse. DPCM, similarly to distortion products measured acoustically, reflects active amplification at the OHCs (Kemp and Brown 1984; Kujawa et al., 1992).

A similar reduction of CAP was observed with ChTX, a toxin also known as a potent blocker of BK channels but acknowledged to be less specific for BK channels than IbTX since it can block also other voltage-dependent K$^+$ channels (Garcia et al., 1991). Apamin, a bee venom toxin specific to SK channels, did not affect CAP indicating that SK channels are essentially restricted to the OHCs medial efferent synapse (Dulon et al., 1998). The results obtained with intracochlear perfusion of ChTX are in good agreement with the recent study of Yoshida et al. (2001) and we are now extending these results to IbTX, a BK specific toxin.

One intriguing question is how the toxins gain access to the IHCs via the perilymph from the scala tympani. There are at least two parameters that give us some information on the access of the toxins to IHCs in vivo. First, it is to be noted that there is a difference of about one order of magnitude between the toxin concentration showing an effect in vivo (0.5 µM) and in vitro when applying the toxin directly to isolated IHCs (0.05 µM). This difference indeed underlined a problem of access when perfusing the toxin via the perilymph. The concentration to obtain an effect in vivo remained in the micromolar range, i.e. low enough to consider the reduction of CAP as a specific block of BK channels. Second, while the toxin has an immediate action on isolated IHCs in vitro, the beginning of reduction in CAP in vivo showed a latency of about 15 min with a maximum inhibition within 15-30 min, indicating a slow and progressive access of the toxin to its targets. We don’t know precisely how the
toxin reached the IHCs. Since we are perfusing the scala tympani, the direct route of access could be the basilar membrane, a structure only composed of one layer of longitudinal cells connected above by an intercellular substance made up of longitudinal filaments. The basilar membrane supports directly the organ of Corti. One can speculate that small peptides such as IbTX bind to the basilar membrane cells and diffuse between cells to reach the IHC synapse.

In the present study, we further demonstrated that the BK-specific toxin IbTX blocked a fast outward current in GP IHCs. The IbTX-sensitive current was activated above -40 mV and was N-shaped suggesting its dependence on a Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels. We have demonstrated in a previous study, using flash UV photorelease of intracellular Ca\(^{2+}\) and patch clamp recordings, that GP IHCs express a Ca\(^{2+}\)-actived K\(^+\) conductance sensitive to ChTX and TEA (Dulon et al., 1995). This is also in good agreement with recent recordings made in developing mice IHCs by Kros et al. (1998). In the present study, the block of the fast outward current by barium, a potent blocker of BK channels (Vergara et al., 1999), also reinforced the idea that the fast current of IHC is due to the activation of BK channels.

We argue therefore that the reduction in CAP by ChTX and IbTX during cochlear perfusion \textit{in vivo} could be explained, at least partly, by an action at pre-synaptic BK channels in IHCs. The mechanism by which the block of pre-synaptic BK channels may alter neurotransmitter release by IHCs however remains to be elucidated. The pronounced reduction in the waveform of CAP, produced by IbTX \textit{in vivo}, seems disproportionate to the partial block of steady-state potassium current shown in IHCs. This discrepancy suggested that the underlying mechanisms may have more to do with timing, or kinetics, of the toxin-sensitive current. We found that the time constant of the membrane outward currents of IHCs increased in the presence of IbTX from an average of 0.6 ms to 5.7 ms. These kinetics of the fast and slow currents are in good agreement with those reported at 20°C by Kros and
Crawford (1990). Assuming that the membrane behaves as a first-order electrical system, the block of the fast BK conductance would result in an increase, by about ten fold, in the corner frequency of IHCs, i.e. to attenuate the IHCs receptor potential at frequencies above 30 Hz. Any alteration that allows a temporal spread of transmitter release by individual hair cells would reduce the amplitude of the CAP (whose amplitude depends on precise coordination of the largest possible number of individual afferent action potentials).

Since BK channels are known to be co-localized with the calcium channels that regulate transmitter release in active zones of lower vertebrate hair cells (see for review Fettiplace & Fuchs, 1999), we hypothesise that a similar mechanism occurs in mammalian IHCs. The fast activation of the outward current and the N-shaped I/V curve serve as a good argument for a tight coupling with a Ca\(^{2+}\) influx. *In vivo* during cochlear perfusion, the block of the fast repolarizing BK current by IbTX may maintain these IHCs micro-domains depolarised, breaking the driving force for Ca\(^{2+}\) entry, and as a result diminish Ca\(^{2+}\)-dependent neurotransmitter release. Such decrease of transmitter release by pre-synaptic block of BK channels has been proposed in nerve-muscle synapses (Patillo et al., 2001).

The assumption of expression of BK channels in IHCs was further reinforced in our study by the pattern of rSlo mRNA expression that we found in rat inner ear. Indeed, we show for the first time that rSlo antisense riboprobes hybridized strongly in IHCs and in spiral ganglion and to a lesser extent in OHCs. A similar pattern of expression of the Slo protein was observed by immunocytochemistry in guinea pig organ of Corti. These results are in agreement with recent studies showing the expression of Slo mRNA by RT-PCR in the rat cochlea (Brändle et al., 2001) and the immunolocalisation of BK channels in murine spiral ganglion neurons (Adamson et al. 2002). Furthermore, the pattern of hybridization of antisense riboprobes derived from the carboxy-terminal domain of rSlo that we observed in our study resembles the pattern observed in the chicken's cochlea (Rosenblatt et al., 1997). Further
experiments are now needed to identify whether mammalian cochlea hair cells express multiple and specific BK splice variants as in the chick cochlea.

Our results for in situ hybridization and immunocytochemistry suggest that Slo channels underlie the IbTX-sensitive fast outward current recorded in isolated IHCs. The expression of Slo in spiral ganglion neurons also suggest a role for BK channels in the fast repolarization of action potential in auditory nerve fibres. We believe however that the reduction in CAP that we observed during intracochlear perfusion of IbTX and ChTX in vivo was not due to an effect at the post-synaptic nerve fibres. First, the immunolocalisation of BK proteins did not reveal any expression in the peripheral nerve fibers and we believe that the soma of the spiral ganlion neurons would have a poor access to the toxin since they are tightly surrounded by a myelin shield. Furthermore, we think that the block of BK channels at the post-synaptic level would on the contrary increase excitability in the nerve fibers by keeping membrane potential near their action potential threshold. On the other hand, we have observed that Slo channels are also expressed in stria vascularis suggesting the presence of BK channels. This is in good agreement with previous electrophysiological recordings (Takeuchi et al., 1992). In our intracochlear perfusion experiments in vivo, we also need to consider the possibility that BK toxins alter the standing or evoked current through IHCs by changing the endocochlear potential (EP). However, such a mechanism appears unlikely because CM, which is sensitive to change in EP, remained essentially unaffected during the perfusion of the BK-specific toxins. It has to be noted that the expression of Slo by OHCs, which was however much less than that of IHCs, is consistent with electrophysiological recordings showing the expression of Ca\(^{2+}\)-activated K\(^+\) currents in these cells (Housley & Ashmore, 1992). The absence of effect on DPCM in our study however suggests that IbTX-sensitive channels in OHCs have a minimal influence on the cochlear amplifier.
Based on these results we hypothesise that a fast BK conductance regulates neurotransmitter release at pre-synaptic active zones of mammalian IHCs. These BK channels with fast kinetics, presumably composed of Slo subunits, allow IHCs to function as high-frequency signal transducers (Palmer & Russel, 1986; Kros et al., 1998). While we were finishing the writing of our manuscript, an *in situ* hybridization study showing BK mRNA expression in the rat cochlea, and particularly in IHCs, was released by Langer et al. (2003), in agreement with our study.
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ABBREVIATIONS

BK channels = large conductance calcium-activated potassium channels

CAP = compound action potential

ChTX = charybdotoxin

CM = cochlear microphonics

DPCM = distortion products in cochlear microphonics

IbTX = iberiotoxin

IHC = inner hair cell

GP = guinea pig

nAChR = nicotinic acetylcholine receptor

OHC = outer hair cell

SK channels = small conductance calcium-activated potassium channels

TTX = tetrodotoxin
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FIGURE LEGENDS

Figure 1: Sound-evoked cochlear potentials recorded in the guinea pig cochlea

A) A raw trace of an electrical compound response recorded from the scala vestibuli during a tone burst stimulation (8 kHz, 60 dB SPL) is shown.

B) The separation of the compound auditory potential (CAP) and the cochlear microphonic (CM) has been performed by low-pass (lower trace) and high-pass filtering, respectively (upper trace, \( F_c = 3\) kHz). CAP represents the summed, synchronous activity of auditory nerve fibres while CM is the AC receptor potential generated by the hair cells.

C) In the lower diagram, the FFT of the CM response evoked by the simultaneous application of two pure tones \( (f_1=8\) kHz and \( f_2=9.68\) kHz) and its distortion products (DPCM: \( 2f_1-f_2=6.32\) kHz) is shown. DPCM is believed to result from the active mechanical transduction mechanism occurring at the OHCs level.

Figure 2: Intracochlear perfusion of TTX blocks CAP

Traces a,b,c,d are compound responses to a 8 kHz tone burst (50 dB SPL) (gray area) with superimposed CAP waveform (black), recorded at different times, during a continuous perfusion of HBSS or HBSS+TTX (3 µM) in the guinea pig scala tympani (drug application indicated by the gray box). The lower diagram shows the changes in CAP amplitude over time. Note the rapid decrease of CAP amplitude during TTX perfusion, without recovery afterward, while the CM remains unchanged. In this figure and in the following, the solid curve represents successive polynomial fitting of the data points (O).
Figure 3: Intracochlear perfusion of ChTX

Same representation as in Fig. 2 during a continuous perfusion of HBSS (H) or HBSS+ChTX (2 µM) in the guinea pig cochlea (drug application time is indicated by the gray box). Sound stimulation was set at 8 kHz and 60 dB SPL. Note the reversible changes in CAP amplitude and the lack of change in CM amplitude.

Figure 4: Intracochlear perfusion of IbTX

Same representation as in Fig. 2 and 3 during successive perfusion with HBSS (H) and HBSS+IbTX in the guinea pig cochlea (1 µM; drug application time is indicated by the gray box). Sound stimulation was set at 8 kHz and 60 dB SPL. Note the reversible changes in CAP amplitude and the slight increase in CM amplitude at times of maximum suppression of CAP (c, h).

Figure 5: Dose-dependent effect of IbTX at different sound pressure levels

A) Changes in CAP amplitude during perfusion with IbTX at different concentrations (HBSS perfusion was maintained before, after and in between the IbTX perfusions 1, 2, 0.5 and 5 µM) and for 4 levels of tone burst stimulation (8 kHz at 30, 50, 70, 90 dB SPL).

B) Reduction of CAP as a function of sound intensity.

C) Reduction of CAP as a function of IbTX concentration. Data were best fitted with a sigmoidal Hill equation with Hill coefficient set to 1. An IC50 ranging between 0.4 to 1 µM for 70 and 90 dB, respectively, resulted from the fit.
Figure 6: Intracochlear perfusion of 1 µM IbTX reversibly reduces the compound action potential (CAP) without changing the output of the distortion products in cochlear microphonics (DPCM). As described in figure 2, cochlear potentials (CAP, CM and DPCM) were evoked by the simultaneous application of two pure tones (f1 = 8 kHz) and (f2 = 9.68 kHz) at various sound levels from 40 to 80 dB SPL. The two vertical lines indicate the 15 min perfusion of IbTX.

Figure 7: Example of IbTX-sensitive outward currents recorded from an isolated IHC

The IHC shown in the photograph was held at -60 mV and was depolarized in 10-mV increments from -80 mV up to 50 mV (A). The cell was then subjected to a similar protocol in presence of IbTX (50 nM) (B). The IbTX-sensitive current was obtained by subtracting A-B traces (C). The current-voltage relationships of the IbTX-sensitive current measured at the peak-current (Δ) and the plateau (O) are plotted in D. The graph D in insert plots the time-constant of activation measured in C as a function of the amplitude of voltage-step. Voltage errors attributable to series resistance and liquid junction potential were corrected in D.

Figure 8: In situ hybridization for Slo mRNA in the cochlea (A) and in the vestibular organ, (B).

A) Expression of Slo mRNA indicated by the small white dots (silver grains under dark field microscopy) was mainly observed in IHCs and spiral ganglion (SG). Some labelling was also seen in the area of the OHCs (possibly Deiters cell) and the stria vascularis.

B) Expression of Slo mRNA in a crista ampullaris indicated a strong labelling in the sensory hair cell epithelium.
**Figure 9: Immunoreactivity of BK channels in the whole mount surface preparation of the guinea pig organ of Corti**

In A, the figure displays the immunolabelling of a surface preparation of a second turn of the guinea-pig cochlea observed at low magnification (bar = 100 µm). In B, the same preparation as in A observed at higher magnification (bar = 40 µm). Note a strong labelling in the IHCs region and a moderate labelling in the three rows of OHCs. In C and D, observation at high magnification in the basal turn of the organ of Corti (bar = 20 µm).

In D, seen from underneath the organ of Corti, the labelling of the cell bodies of two IHCs can be clearly observed. The anti-BKCa is a polyclonal antibody against the subunit C-terminal part (residues 1098-1196).

**Figure 10: Immunoreactivity of BK channels in cryo-sections of the guinea pig cochlea**

A) Immunoreaction in the organ of Corti (OC) and spiral ganglion neurons (SG) observed at low magnification. Note the absence of immunoreactivity in the nerve fibers (arrow).

B) Immunoreaction in the organ Corti was essentially noted in inner hair cells (IHC), outer hair cells (OHCs) and Deiters cells.

C) and D) displays the immunoreaction at high magnification in IHCs from second and first turn of the cochlea. The arrows indicate a stronger labelling at the base of the IHCs and the presence of dot-like immunoreactions.

The scale bar in B corresponds to 100 µm in A, 40 µm in B and 10µm in C and D.
Figure 2

![Figure 2](image_url)

**Figure 2**

**CAP amplitude:** µV

- **a**
- **b**
- **c**
- **d**

**50 µV**

**10 ms**

**CAP amplitude:** µV

**0**

**30**

**60**

**90**

**120**

**150**

**HBSS**

**TTX**

**HBSS**

**minutes**

**0**

**25**

**50**

**75**
Figure 3
Figure 5

A

B

C

Block of CAP (µV)

Level of tone burst (dB SPL)

BLOCK of CAP (µV)

IbTX (µM)
Figure 6

CAP amplitude: µV

DPCM amplitude: dB re 1 µV

F2 CM amplitude: dB re 1 µV

F1 CM amplitude: dB re 1 µV
Figure 7

A. Control

B. BtX

C. Current vs. Time (ms)

D. Current vs. Membrane Potential (mV)

IHC
Figure 8
Figure 10

A B

OC SG

DC OHCs IHC

C D

synaptic area

D

synaptic area