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ENDOTHELIN-1 INHIBITS OUTWARD POTASSIUM CURRENTS IN MOUSE OUTER SULCUS CELLS

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Abstract – The outer sulcus cells are epithelial cells covering the luminal side of spiral sulcus of cochlea. It has been suggested that outer sulcus cells contribute to cation absorption from the lumen of the cochlea. We investigated the electrical properties and the effects of endothelin-1 (ET-1) on the outward potassium currents in mouse outer sulcus cells using a whole-cell patch clamp technique. The cell capacitance was 3.16±0.66 pF (n =35) and the resting membrane potential was -98.4±1.6 mV (n=6) in extracellular fluid bath solution. The outward K+ currents were activated by depolarizing pulses more positive than -60 mV, and was sensitive to TEA (10 mM). Tail current analysis revealed that it was primarily K^+ selective. Application of ET-1 caused a decrease of outward potassium currents within seconds, whereas treatment with BQ123, a competitive inhibitor of the ET type-A receptor, counteracted the inhibitory effect of ET-1. These results suggest that ET-1 inhibits outward potassium currents through the activation of ET type-A receptor. ET-1 may play an important role in maintaining the ionic homeostasis of endolymph.

Key words: BQ123, cochlea, ET-1, mouse, outer sulcus cells, outward potassium currents, whole cell patch clamp techniques

INTRODUCTION

The outer sulcus cells are epithelial cells lining the luminal side of spiral sulcus of cochlea (3,22). It has been recently shown that the outer sulcus epithelial cells contribute to cation absorption from the lumen of the cochlea (17). Unlike the sensory hair cells, the outer sulcus cells provide a parasensory pathway in the cochlea to maintain the ionic homeostasis of endolymph. It has been suggested that Na^+ and K^+ are taken up across the apical membrane by the nonselective cation channels of the outer sulcus cells. Na^+ is removed by the Na^+, K^+-ATPase and K^+ diffuses across the basolateral K^+ channel (1). Modulation of ion channel activity may play a significant role in the homeostasis of inner ear fluids. It was reported that the epithelium regulates K^+ reabsorption by apical purinergic receptors (16).

Endothelin-1 (ET-1) originally characterized as a vasoconstrictive peptide has been revealed to have many different biological functions, such as to be a local hormonal regulator of pressure, fluid, ions and neurotransmitters in inner ear (8,9,21). The effects of ET-1 are mediated via two receptor subtypes, ET type-A and ET type-B receptors (ETAR and ETBR), which are coupled to Gq- and Gi-proteins. Previous studies have suggested that endothelins act as modulatory peptides, possibly interfering with nitric oxide, prostaglandins, and atrial natriuretic peptide in the lateral cochlear wall (4). There were also some reports proposing that ET-1 may play an important role in the regulation of inner ear pressure, fluid volume, and ion balance (10). However, the mechanisms of the action of ET-1 are not fully understood. A variety of electrophysiological effects have been reported following the stimulation of the ET-1. It was previously suggested that ET-1 increases

Abbreviations: BK, maxi-K^+ ; **EP**, endocochlear potential; **ET-1**, Endothelin-1; **ETAR**, endothelin type A receptor; **ETBR**, endothelin typeB receptor; **NSt**, nonstrial tissues; **NSC**, nonselective cation channel ; **RT-PCR**, reverse transcription-polymerase chain reaction

voltage-dependent Ca^{2+} current in rabbit cardiac myocytes (15), stimulates L-type Ca^{2+} currents by activation of protein kinase C in rat ventricular myocytes (7,13,15,24), and activates the Na^+/H^+ exchanger (14). Recently, ET-1 was reported to activate nonselective cation currents in human bronchial smooth muscle cells, inhibits src family kinases-mediated epithelial Na^+ channel, and augments the delayed rectifier postassium current by ETAR in guinea pig atrial myocytes (6, 19,20).

As mentioned above, ET-1 was widely expressed throughout the cochlea (4,10,21,26). Potassium channels were involved in the pathological process of cation absorption from the lumen of the cochlea. Regulation of their activity influences ionic homeostasis of endolymph. The mechanisms of the action of ET-1 remain unclear. Whether ET-1 has a regulatory effect on the potassium channel currents in the outer sulcus cells is still unknown. The objective of this work was to determine whether ET-1 affects K^+ currents and to identify the current components involved. We used whole-cell patch clamp technique to examine the effect of ET-1 on the potassium channels in freshly isolated outer sulcus cells. We further tested if the effect of ET-1 on the potassium channels was related to its receptors on the cell membrane. We present evidence to reveal the importance of ET-1 in maintaining the homeostatic environment of cochlea.

MATERIALS AND METHODS

Tissue preparation

Adult KM mice at the age of about 30 days were used in this study. Mice were decapitated, the temporal bone was removed, and spiral ligament from the 2nd and 3rd turns was dissected at 4°C as described previous (17). The stria vascularis was removed. A piece of spiral ligament containing outer sulcus cells was folded into a loop so that the outer border was formed by the apical membrane of the outer sulcus cells. This tissue was mounted in a bath chamber on an inverted microscope (Nikon, Diaphot). The edge of the fold was brought into focus so that gigohm seals could be formed between the patch pipette and the apical membrane under visual control. The procedures concerning animals reported in this study were approved by the Animal Care and Use Committee at the Huaxi Medical School of Sichuan University.

Electrophysiology

Conventional whole cell patch clamp techniques were used in this study. The chamber was placed on the stage of an inverted Olympus IX70 microscope (Olympus, Japan). The chamber was filled with approximately 3 ml of saline. In most experiments outer sulcus cells were maintained at -80 mV of voltage. In a standard protocol, cells were stepped to a series of voltage from -70 to 80 mV with an increase of 10 mV for 400 ms and then returned to -70mV. Other protocols in whole cell voltage clamp experiments were also used. Briefly, electrode tips were filled with approximately 300 µl of intracellular solution and back filled with intracellular pipette solution containing dimethyl sulfoxide. Patch electrodes were pulled on a P-97 puller (Sutter Instruments, Novato, California). When filled with appropriate pipet solution, they had a resistance of 2 to 6 MΩ. The seal resistance between the patch and pipette was usually 2 to 5 GΩ. Data were collected using an Axopatch 200B voltage amplifier. All the experiments were performed at room temperature. The liquid junction potential between bath and internal solution was corrected. Series resistances were compensated by 80%. During the recording, the change of series resistance was negligible.

Solutions and drugs

The physiological (external) solution contained (in mM): 150 Na-Gluconate, 1.0 $MgSO_4$, 1.6 K_2HPO_4, 0.4 KH_2PO_4, 4.0 Calcium –Gluconate, pH 7.4 (buffered with NaOH). The pipette solution contained (in mM): 150 K-Gluconate, 1.0 $MgSO_4$, 10 HEPES, 1 EGTA, pH 7.4 (buffered with KOH). Pipette solution was frozen at -20°C and filtered prior to use .The ET-1 (Sigma) was dissolved as stocks in Ringer solution and kept at -20°C. Before experiment, the stocks were diluted in Ringer solution at the tested concentrations. In some experiments, influences of BQ123(Sigma), an ETAR antagonist, on the ET-1-induced changes in outward potassium currents were examined.

Reverse transcription-polymerase chain reaction (RT-PCR)

Transcripts for ET-1 and ETAR were assayed by RT-PCR. Total RNA was isolated from the spiral ligament of the mouse cochlea using Trizol reagent (MRC USA). RNA was reverse transcribed into cDNA using the Revert Aid™ First Strand cDNA Synthesis Kit (MBI Fermentas Inc.) with addition of random hexamer primers. Briefly, 5µl of purified RNA was used as template for cDNA synthesis in the presence of 1µl M-MLV reverse transcriptase (200U), 1µl of random hexamer primer, 4 µl of 5×reaction buffer, 2µl of hexamer 1×(Roche), 2µl of dNTP mix (10mM each), and 6µl of RNAse-free water. After incubation for 60 min at 42°C, the reverse transcriptase was inactivated at 70°C for 10 min, and cDNAs were stored at -20°C until further analysis. The sequence of the primers was based on the known sequences in the coding region of the mouse and human.

ET-1, forward primer: 5'-TTCGTGACTTTCCAAGGAGC-3'and reverse primer 5'-GTCCATCAAGGAAGAACAGG-3' (a 147bp product will be generated).

ETAR, forward primer 5'–CTCCATCTGGATTCTTTTCCTT-3' and reverse primer 5' -CTTGGTAAAACTCCATGAACT-3' (a 137 bp product will be generated).

GAPDH, forward primer 5'-TGGGTGTGAACCACGAGAA-3' and reverse primer 5'-GGCATGGACTGTGGTCATGA-3' (a 143 bp product will be generated).

The PCR was performed as follows: one denaturation cycle for 2 min at 94°C; forty amplification cycles consisting of denaturation for 20 sec at 94°C, annealing for 30 sec at 55°C, and extension for 40 sec at 72°C; and one extension cycle for 5 min at 72°C. PCR products were analyzed by horizontal electrophoresis in 1.5% agarose gels and visualized by ethidium bromide.

Data analysis

Data were analyzed using Clampfit (version 8.1, Axon Instruments) and Excel (Microsoft). Data are expressed as the mean ± standard error. The paired Students' t-test was used to determine the effects of drugs. Increases or decreases in outward potassium currents were considered to be significant at a value of $P<0.05$.

RESULTS

In the present study, the whole cell K^+ currents of the outer sulcus cells in freshly dissected spiral ligament of the cochlea were examined using patch clamp recording. The results were based on observations made from 46 outer sulcus cells from freshly dissected mouse inner ear. Whole cell capacitance was 3.16±0.66pF (n=35). Fig.1 showed a representative family whole cell currents. The currents were recorded by holding a cell at -80 mV, which is near the reversal potential of K^+ channel with low K^+ bath solution, and with clamping voltage from -70 mV to 80 mV. In this range of membrane potentials, outward currents were elicited by depolarization to-70 mV for 400 ms at the holding potential of -80 mV. It appeared that whole cell currents were outwardly rectified and slowly initiated.

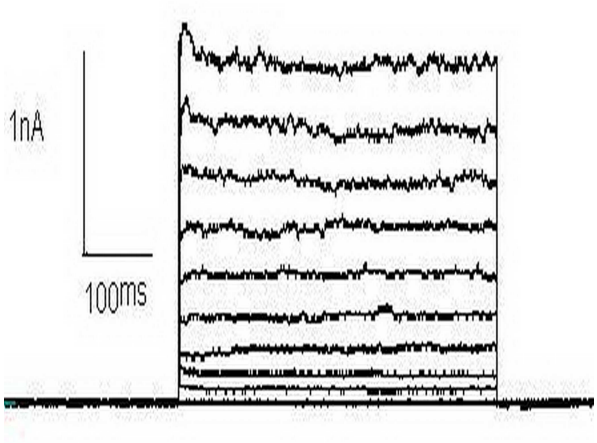

Figure 1. Typical current responses after a voltage step from -70 to +80 mV in an outer sulcus cell. Whole-cell outward currents were evoked by a holding potential of -80 mV for a duration of 400 ms with voltage steps between -70 to +80 mV.

No rundown of the current responses was observed. The current also showed slightly rectifying, outwardly directed I–V relationships with a markedly negative reversal potential, -98.4±1.6 mV (n=5), close to the calculated Nernst equilibrium potential for K^+ at physiological relevant intra- and extra-cellular concentrations. As shown in Fig. 2, the outward currents were largely reduced in the presence of 10 mM TEA in the external solution. In some of the cells (n=7), the fast inactivating potassium currents were observed. The currents were activated rapidly (10 ms) to a peak (Ipk) and subsequently inactivated to an outward current level at the end of the pulse (Ilate) (Fig. 3A). The fast inactivating outward currents were suppressed by holding cells at -40 mV with various voltages between -20 and +70 mV, indicating that the outward current was the fast inactivating potassium currents (Fig. 3B).

Exposure to ET-1 for 180 s significantly decreased the steady-state outward current at +80 mV by 40.13 % in 5 cells (from 159.74 ±35.96 to 95.63±37.41 pA/pF, $p < 0.05$). The effect of ET-1 on the current density-voltage relation was examined. In the presence of 10 nM ET-1, outward potassium currents were inhibited in a voltage-dependent manner (Fig 6). Typical current response of reduction in control by ET-1 was reversed by bath application of 1µM BQ123 from a single outer sulcus cell (Fig 4). A typical effect of ET-1 treatment for 2 min on outward currents is illustrated in Fig 5. The amplitude of the outward current was declined in less than 1 min after switching to ET-1 conditions. Similar results were obtained in 4 other cells.

The inhibition of outward potassium currents by 10 nM ET-1 was abolished by the ETAR-selective antagonist BQ123 (Fig. 6). In the presence of 1µM BQ123, the outward potassium currents at +80 mV potential after addition of 10 nM ET-1 was only 25.33 % (n = 4) .These findings suggest that ET-1 inhibits outward potassium currents through the activation of ETAR. We tested for the presence of transcripts for ET-1 and ETAR in the nonstrial tissues (NSt) of the cochlear lateral wall. As shown in Fig. 7, we demonstrated that both ET-1 and ETAR mRNAs were expressed in the NSt of the cochlear lateral wall. We used heart samples, which have been previously shown to express ET-1 and ETAR, as positive controls. GAPDH was examined as a housekeeping gene.

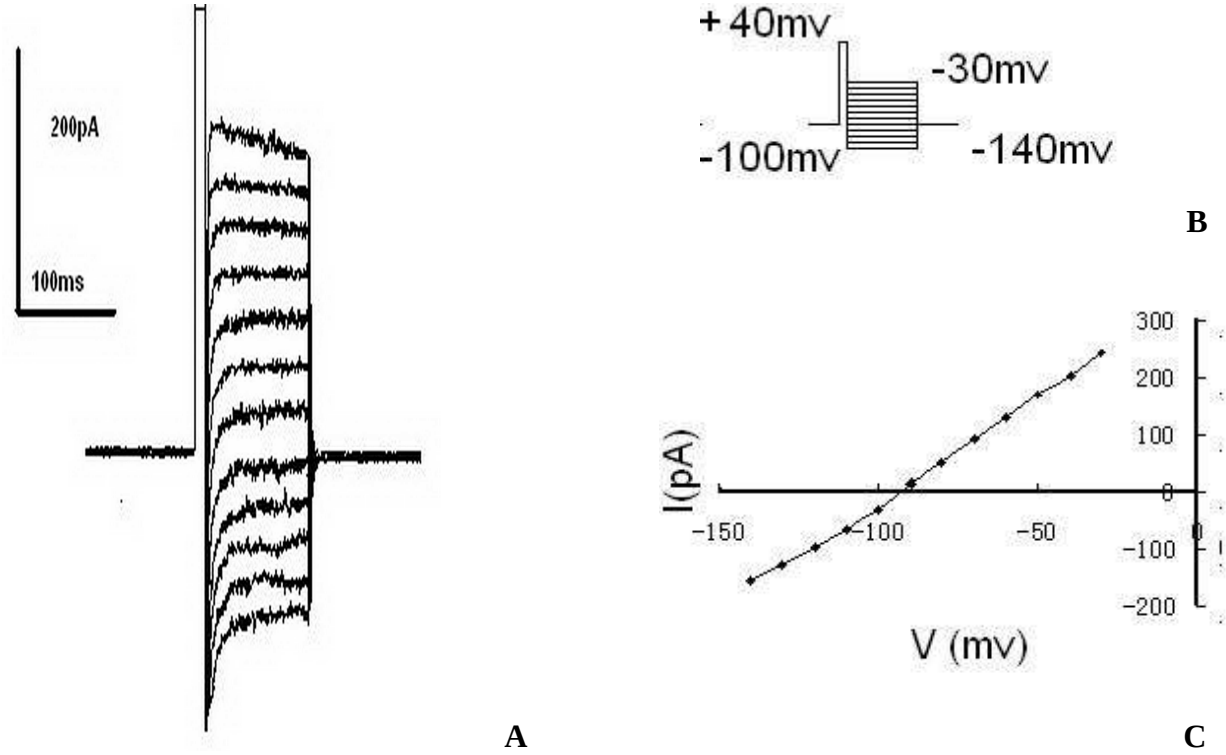

Figure 2. (A) The tail currents from outer sulcus cells in mouse. (B) The voltage steps from -140 to -30 mV and holding potential at -100 mV were used to determine the reversal potential of outward currents. (C) Current–voltage relationship showed that the reversal potential of the outer sulcus cells was 98 ±1.6mV (n=5).

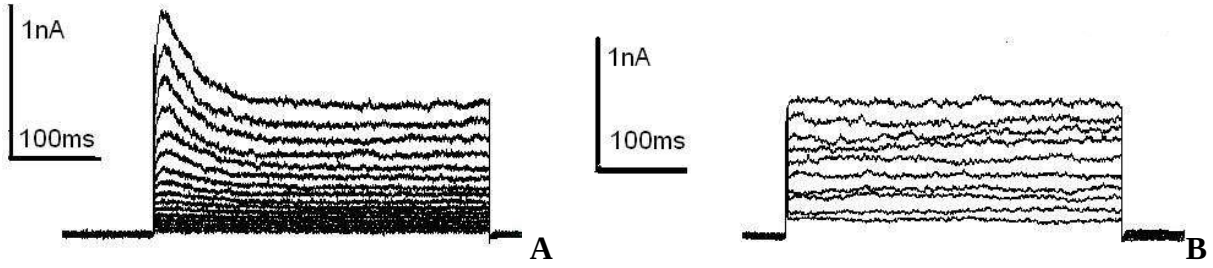

Figure 3. (A) The fast inactivating potassium currents in one of the outer sulcus cells. (B) The fast inactivating outward current was suppressed by holding cells at -40 mV with voltages between -20 and +70 mV.

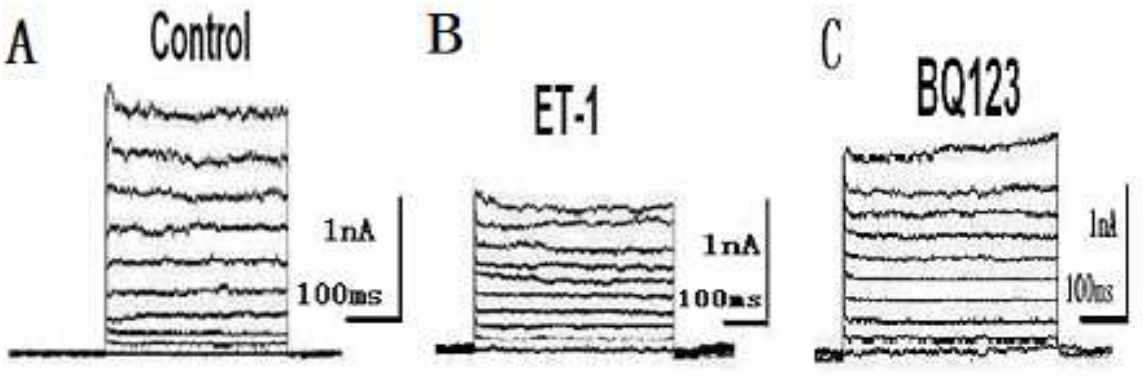

Figure 4. (A) Outward potassium currents activated by the voltage from -70 mV to 80 mV before application of ET-1. (B) Outward potassium currents activated by the voltage from -70 mV to 80mV after application of ET-1. ET-1 decreased outward potassium currents. (C) Reduced outward potassium currents reduced in control by ET-1 was reversed by bath application of 1μM BQ123.

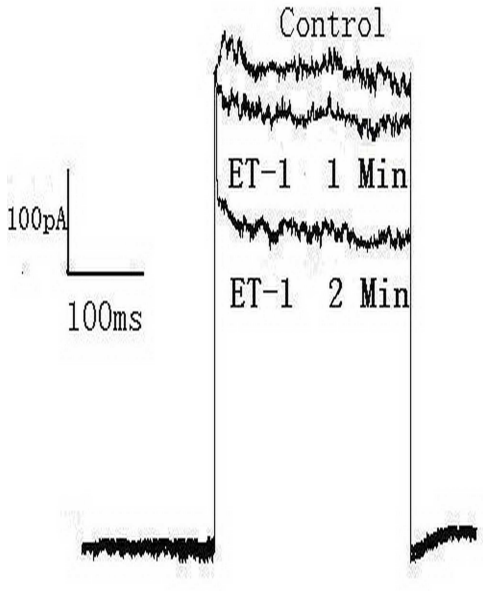

Figure 5. ET-1 decreased the amplitude of the outward potassium currents. ET-1 at the dose of 10nM was added to the bath solution for 2 min, the outward potassium currents were recorded as described above. Currents evoked to test potentials to +60 mV are shown for clarification. Currents shown include control and 10 nM ET-1.

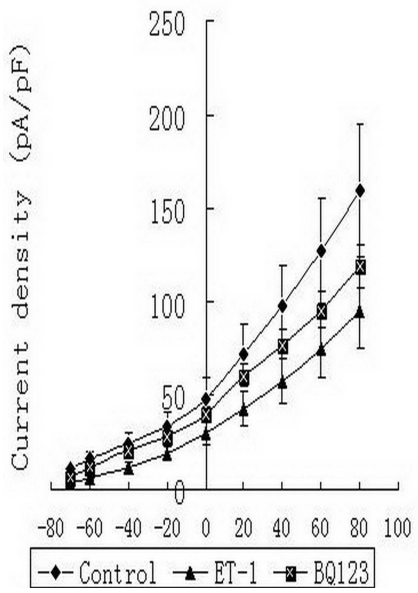

Figure 6. The current density–voltage relationship in the presence of ET-1 and BQ123. Reduction in the outward potassium current produced by ET-1(10 nM) was partially reversed by BQ123 (1μM) ($p<0.05$).

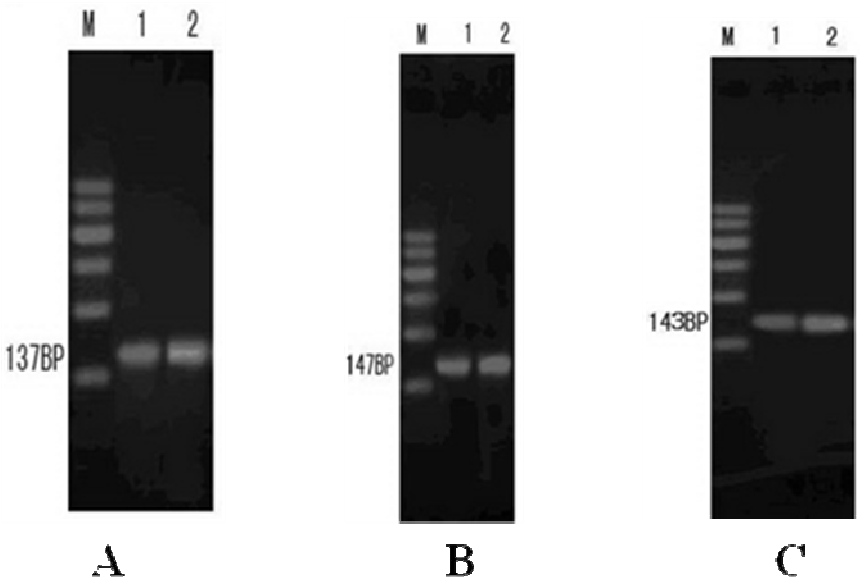

Figure 7. A Gel electrophoresis of RT-PCR products from mice spiral ligament. Amplified fragments were of expected size, 147 and 137bp for the ET-1 and ETAR gene (A, B), respectively. Amplification of a 143 bp fragment of GAPDH cDNA was shown as housekeeping controls (C). M: marker; lane 1: heart tissues; lane 2: nonstrial tissues of the cochlear lateral wall.

DISCUSSION

The ionic composition of the endolymph in cochlea duct was characterized by high potassium and low sodium levels. A high positive endocochlear potential (EP) was also found in endolymph. Maintenance of the unusual ionic composition of endolymph in the cochlea is essential to the conduction of sound. The outer sulcus epithelium was recently shown to absorb cations from the lumen of the gerbil cochlea (17).

The present study demonstrated that K^+ was involved in the outward current in outer sulcus cells of mice. It was found that the predominant voltage-activated currents in outer sulcus cells were K^+ selective and outward rectifying. We further studied the effect of K^+ channel blockers on the current. External application of 10 mM TEA blocked this current. TEA is effective in reducing the amplitude of the K^+ current from outside of the membrane.

It has been reported that ET-1 was expressed extensively in the cochlea including the outer sulcus cells (4,10). It has also been found that ET -1 receptor subtype ETAR was expressed in stria vascularis of mice (5). In this study, we showed that ET-1 was able to inhibit potassium currents in the acutely isolated outer sulcus cells. The channels carrying these currents interacted with ET-1 in a voltage-dependent manner. Furthermore, ETAR-selective antagonist BQ123 rapidly, reversibly and voltage-dependently abolished the inhibitory effect of ET-1 on the outward potassium currents, suggesting that the response to ET-1 at the apical side of outer sulcus cells was functionally mediated by ET receptors.

Effects of ET-1 on ion currents have also been reported in other cell types. Previous reports suggest that ET-1 can acutely inhibit ATP-sensitive potassium of retinal pericytes (12). In contrast to this result, ET-1 activates large-conductance K+ channels of rat lactotrophs and the delayed rectifier potassium current in guinea pig atrial myocytes, augments the delayed rectifier potassium current in guinea pig atrial myocyte by ETAR (11,19). Thus, there appear to be different functions of ET-1, depending on cell and channel types. Although physiological and pathophysiological significance of the inhibition of potassium currents by ET-1 is still unclear in the present investigation, it may play an important role in the modulation of transepithelial K^+ transport. The inhibition by ET-1 on the outer sulcus cells potassium currents may affect the resorption of K^+. Accumulation of K^+ in the endolymph may cause a change of endocochlear potential. We could reasonably speculate that ET-1 influences the ionic homeostasis of endolymph by regulating the outward K^+ current in outer sulcus cells.

Outer sulcus cells actively absorb cations by cellular mechanisms and serve as parasensory pathways to regulate K^+ efflux. Because inhibition of the K+ channels is considered to reduce the resorption of K^+, partial inhibition of outward current by ET-1 may lead to the accumulation of K^+. It can be expected that inhibition of this current will lead to an increase of endocochlear potential and may be one of the cause of the hydrolabyrinth.

Several types of ion channels, including nonselective cations, maxi-K^+ (BK) and small K^+ channels, were found in the apical membrane of outer sulcus cells of Gerbils (17). Different types of ion channels might coexist in the outer sulcus cells and open under different circumstances. In this study, the electrophysiologic measurements used in this report could not identify the corresponding channels type. BK channel is the major K^+ channel found in the apical side of outer sulcus cells. BK and nonselective cation channel (NSC) might be the important contributors of transepithelial K^+ and Na^+ transport (1,2,17). In this study, the corresponding I–V relationship of the current was an approximately linear between -30 mV and +80 mV, its sensitivity to TEA, its ion-permeability properties, suggest that this outward current may be mediated by BK channel that is activated by a calcium-dependent intracellular cascade process and/or some uncertain channel types.

Our results suggest that ET-1 can partially inhibit the outward potassium currents in the outer sulcus cell through the activation of ETAR. It has been shown that the binding of ET-1 with the ETAR activates phospholipase C, leading to the formation of inositol 1,4,5-trisphosphate and diacylglycerol (18). The release of ET -1 from the vascular endothelial cells activates ETAR that predominates on the underlining vascular smooth muscle cells. The precise mechanisms of ET and ETAR interaction in outer sulcus cells are still unclear. Whether it is through the same signal pathway requires to be clarified by further experiment. The effects of ET-1 are mediated through two receptor subtypes, ETAR and/or ETBR. We did not examine the effect mediated by ETBR in our work. Whether ET-1 affects K^+ currents of outer sulcus cells through ETBR also requires to be clarified in future study.

Recently, there was another research finding that the activity of the ET system of the cochlea stepped up significantly in the process of the noise-induced injury of inner ear (25). It was proposed that ET may play an important role in the process of the cochlear microcirculation disorder caused by noise. Therefore, the abnormality of ET system in the cochlea may involve in the pathogenesis of certain diseases. The present study may benefit the understanding of the mechanism of the hydrolabyrinth and hearing loss**.** Further investigation of the regulatory molecules and intracellular signaling systems needs to be performed to explore the mechanisms by which ET-1 modulates the activity of outward currents in outer sulcus cells.

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