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Presence of Adrenergic Receptors in Rat Endolymphatic Sac Epithelial Cells

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Abstract Intravenous application of catecholamines produces a depression in the endolymphatic sac direct current potential (ESP) and increases endolymphatic pressure via the β-adrenergic receptor (AR) in guinea pigs, suggesting that catecholamines play a role in the endolymphatic system. However, the localization of ARs in the endolymphatic sac (ES) is still undetermined. The presence of ARs in the rat ES was investigated by reverse transcriptase-polymerase chain reaction using laser capture microdissection (LCM) and immunohistochemical analysis. Expression of α_{1A}-, α_{1B}-, α_{2A}-, α_{2B}-, β_1-, β_2- and β_3-ARs was observed in LCM samples of ES epithelia. Immunohistochemical analysis using specific antibodies showed immunofluorescence of β_2- and β_3-ARs in epithelial cells of the ES intermediate portion, and no specific staining results were obtained for α_1-, α_{2A}-, α_{2B}- and β_1-ARs. The presence of β_2-AR with no clear immunostaining of β_1-AR in ES epithelial cells is in accordance with previous electrophysiological and pharmacological results, which suggests that β_2-AR mediates the action of catecholamines on the ESP. The presence of β_3-AR in the ES epithelial cells and its absence in the stria vascularis implies that β_3-AR plays a specific role in the ES.

Keywords Adrenergic receptor · Endolymphatic sac · Stria vascularis · Laser capture microdissection · RT-PCR · Immunohistochemistry

Introduction

The endolymphatic sac (ES) is located inside the temporal bone and is a part of the membranous labyrinth that contains the cochlea, vestibular organs and semicircular canals. The ES consists of an intraosseous part, which is connected to the vestibule via the endolymphatic duct and is surrounded by the vestibular aqueduct, and an extraosseous part, which is located distal to the external aperture of the vestibular aqueduct; and its superficial region is layered with the dura mater (Lo et al. 1997). According to Guild (1927), the ES is divided in to three parts: the proximal, intermediate, and distal. The intermediate portion is thought to be involved in endolymph absorption. Surgical blockage of the ES and endolymphatic duct causes endolymph accumulation in the cochlea and vestibule as so-called endolymphatic hydrops (Kimura 1967), a characteristic pathological finding in Meniere disease. Endolymphatic hydrops causes in the cochlea deafness and in the vestibule vertigo (Sakikawa et al. 1999; Tonndorf 1976). Endolymph regulation is thus important for hearing and the sense of equilibrium (Couloigner et al. 2004; Takumida et al. 1991). Although the ES is generally accepted to contain an active transport system and may absorb the endolymph (Kimura 1967), no mechanisms underlying ES-mediated endolymph regulation have been established.

Catecholamine hormones are released by the adrenal glands and are involved in the fight-or-flight response to psychological or environmental stressors, among other sympathetic nervous system actions. The action of

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catecholamines is mediated through adrenergic receptors (ARs), which are classified into α_1-, α_2-, β_1-, β_2- and β_3-ARs; and catecholamines play a central role in cardiovascular physiological phenomena. The inner ear is also one of the targets of catecholamines. Electrophysiological analysis revealed that K^+ secretion in strial marginal cells of the stria vascularis of the cochlea was stimulated via β_1-ARs but not β_2-ARs (Wangemann et al. 2000). Confocal immunocytochemistry revealed the localization of β_1-ARs in the strial marginal cells and vestibular dark cells (Fauser et al. 2004). Intravenous application of catecholamines depresses the endolymphatic sac direct current potential (ESP), which is generated by active ion transport and used as an index of ES function (Mori et al. 1990). A study using selective agonists and antagonists has shown that β_2-ARs mediate the action of catecholamines on the ESP (Mori and Uozumi 1991). Isoproterenol has been reported to increase the endolymphatic pressure in guinea pigs via its action on the ES (Inamoto et al. 2009). Despite physiological and pharmacological evidence of β-adrenergic action on the ES, the localization of ARs in the ES has not been investigated. The present study examined the expression and localization of ARs in the ES.

Materials and Methods

Animals and Tissue Preparation

Four-week-old female Sprague-Dawley rats were purchased from Charles River (Yokohama, Japan). This study was approved by the Animal Care and Use Committee of Kagawa University.

For immunohistochemical analysis, rats were deeply anesthetized using diethyl ether. Subsequently, blood was collected and perfused via the left ventricle with a fixative solution [4 % paraformaldehyde in phosphate-buffered saline (PBS)] for approximately 5 min, and then rats were decapitated. The temporal bones were removed, and the ES on both sides, including the surrounding bone tissues, was dissected carefully under a stereomicroscope. The heart, lungs and esophagus were also collected. Samples were fixed in 4 % paraformaldehyde in PBS for 6 h at 4 °C. The temporal bones containing the ES were then decalcified in 0.12 M ethylenediaminetetraacetic acid (EDTA, pH 6.5) at 4 °C for about 14 days. Decalcified temporal bones and other organs were embedded in optimal cutting temperature (OCT) tissue compound (Sakura Fintechnical, Tokyo, Japan). Sections (7–10 μm thick) were cut on a cryostat at −20 °C and mounted on Matsunami adhesive slide (MAS)-coated glass slides (Matsunami Glass, Osaka, Japan).

For laser capture microdissection (LCM), anesthetized rats were exsanguinated via the left ventricle with 70 % ethanol/RNase-free water and decapitated. The ES and/or cochlea within the temporal bone were collected from both sides. The kidneys, heart, aorta, lungs, adipose tissue and stomach were also collected and immediately immersed in liquid nitrogen for use in reverse transcriptase-polymerase chain reaction (RT-PCR). The temporal bones containing the ES and cochlea were fixed in 70 % ethanol/RNase-free water for 6 h at 4 °C and decalcified in 0.12 M EDTA (pH 6.5) including RNAlater (Life Technologies, Carlsbad, CA) for about 7 days at 4 °C. Samples were embedded in OCT tissue compound, frozen in liquid nitrogen and stored at −80 °C.

LCM

LCM was performed as previously described with minor modifications (Akiyama et al. 2008a). Briefly, the entire ES or cochlea in the temporal bone was cut into slices (10–12 μm thick) using a cryostat at −20 °C and mounted on MAS-coated glass slides. Sections were refixed and dehydrated in a stepwise manner for 1 min each in 70, 90 and 100 % ethanol/RNase-free water, followed by 5 min incubation in xylene before being air-dried. LCM was performed using Applied Biosystems Arcturus Capsure HS LCM caps (Life Technologies), and ES epithelia (from the distal to the proximal portion)/stria vascularis in the cochlea were selectively collected from the sections. RNA was isolated from LCM samples using the Applied Biosystems Arcturus PicoPure RNA isolation kit (Life Technologies) in accordance with the manufacturer's protocol. Dissected ES epithelia from both sides were combined to obtain one RNA sample (LCM-ES), and dissected stria vascularis from one side was treated as an RNA sample of the stria vascularis (LCM-SV).

RT-PCR and Sequencing

RT-PCR from LCM-ES and LCM-SV was performed as previously reported (Matsubara et al. 2012). Briefly, RNA isolated from LCM samples was reverse-transcribed into cDNA by incubation with a random primer and Moloney murine leukemia virus (MMLV) reverse transcriptase (Takara Bio, Otsu, Japan). cDNA fragments were amplified by 30 cycles of PCR (94 °C for 60 s, 60 °C for 60 s and 72 °C for 60 s) with Ex Taq DNA polymerase, dNTPs (Takara Bio) and specific primer pairs as shown in Table 1. PCR products were separated by electrophoresis on 1.0 % agarose gels and visualized by ethidium bromide staining. cDNA from the blood was used as a negative control. As positive controls, cDNA from the heart for α_{1A}-, α_{1B}- and β_1-ARs; from the stomach for α_{2A}-ARs; from the kidney for α_{2B}-ARs; from the lungs for β_2-ARs; and from adipose tissues for β_3-ARs were used.

Table 1 Oligonucleotide sequences used in RT-PCR experiments

Target adrenergic receptor	Primer (sense, antisense)	Product size (bp)
α_{1A}	5′-GAATGTCCTGCGAATCCAGT-3′	237
	5′-GATTGGTCCTTTGGCACTGT-3′	
α_{1B}	5′-ATCGTGGCCAAGAGGACC-3′	201 (Myslivecek et al. 2006)
	5′-TTTGGCTGCTTTCTTTTC-3′	
α_{2A}	5′-GGTAAGGTGTGGTGCGAGAT-3′	229 (Zhang et al. 2010)
	5′-CAGCGCCCTTCTTCTCTATG-3′	
α_{2B}	5′-ACACCGTCTTCAACCAGGAC-3′	169
	5′-CCCAGAGAAATGGCCATAGA-3′	
β_1	5′-GCTCTGGACTTCGGTAGACG-3′	248
	5′-ACTTGGGGTCGTTGTAGCAG-3′	
β_2	5′-GAGCACAAAGCCCTCAAGAC-3′	209
	5′-TGGAAGGCAATCCTGAAATC-3′	
β_3	5′-TGCGCCCATCATGAGCCAGTGGTG-3′	550 (Wangemann et al. 2000)
	5′-GCGAAAGTCCGGGCTGCGGCAGTA-3′	
GAPDH	5′-GGTGATGCTGGTGCTGAGT-3′	301
	5′-CAGTCTTCTGAGTGGCATTG-3′	

GAPDH glyceraldehyde-3-phosphate dehydrogenase

Table 2 Primary antibodies for adrenergic receptors used in the study

Antibody	Host/clonality	Dilution	Source
α_1	Rabbit polyclonal	1:200	Thermo Scientific (Barrington, IL)
α_{2A}	Rabbit polyclonal	1:200	Acris Antibodies (Herford, Germany)
α_{2B}	Mouse monoclonal	1:200	Acris Antibodies
β_1	Mouse monoclonal	1:100	New East Bioscience (Malvern, PA)
β_2	Rabbit polyclonal	1:100	GenWay Biotech (San Diego, CA)
β_3	Rabbit polyclonal	1:100	Acris Antibodies

Immunohistochemical Analysis

Herein, we used commercially available antibodies, shown in Table 2 with their dilution rates. Immunostaining was performed as described in our previous study with minor modifications (Akiyama et al. 2008b). Sections were washed with distilled water and PBS containing 0.2 % Tween 20 and then covered with blocking reagent (Dako, Tokyo, Japan) for 30 min; subsequently, specific primary antibodies (Table 2) were applied to the sections, which were incubated overnight at 4 °C. After repeated washing with PBS containing 0.2 % Tween 20, sections were incubated with secondary antibodies, Alexa Fluor-488 goat anti-rabbit IgG or Alexa Fluor-488 goat anti-mouse IgG (1:200, respectively; Life Technologies), for 2 h at room temperature. After repeated washing with PBS containing 0.2 % Tween 20, 4′,6-diamidino-2-phenylindole (DAPI) nucleic acid stain (1:5,000, Life Technologies) was applied for 2 min. Sections were rinsed several times in distilled water, mounted with 50 % glycerol on a slide and then observed under an Olympus (Tokyo, Japan) BX51 light microscope. The intermediate portion of the ES was used for analysis. Positive control experiments were performed using the heart for α_1-, β_1- and β_3-ARs; with smooth muscles in the esophagus for α_{2A}- and α_{2B}-ARs; and with the lung for β_2-AR antibodies. Negative controls were treated similarly but with omission of the primary antibodies. To confirm the accuracy and reproducibility of this procedure, 15 rats (30 ESs) were included in the study.

Results

RT-PCR and Sequencing

After PCR amplification from LCM-ES with each primer set, DNA bands of α_{1A}-, α_{1B}-, α_{2A}-, α_{2B} -, β_1-, β_2- and β_3-ARs at the expected sizes were observed on an agarose gel (Fig. 1). DNA products from LCM-SV, shown in Fig. 2, indicated expression of β_1- and β_2-AR but no expression of β_3-AR in the stria vascularis. Positive and negative controls validated the appropriate sensitivity and specificity of the experiment (data not shown).

Immunohistochemical Analysis

Strong expression of β_2-ARs was observed in epithelial cells of the intermediate portion of the ES (Fig. 3). β_3-AR

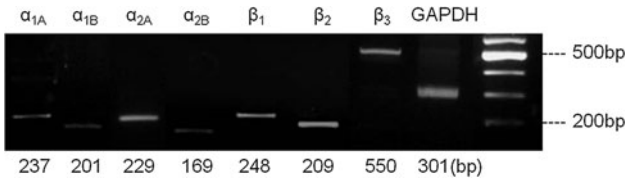

Fig. 1 Agarose gel electrophoresis of PCR-amplified products from the ES. Bands for α_{1A}-, α_{1B}-, α_{2A}-, α_{2B}-, β_1-, β_2- and β_3-ARs are detected at the expected sizes in mRNA isolated from the ES epithelia. ARs are indicated by *numbers*. *GAPDH* glyceraldehyde-3-phosphate dehydrogenase

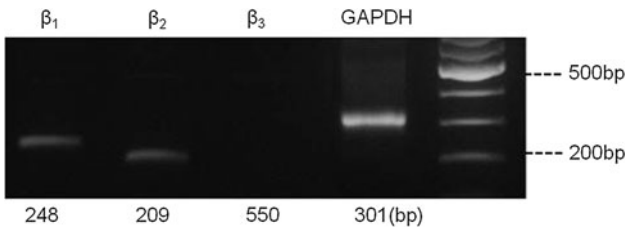

Fig. 2 Agarose gel electrophoresis of PCR-amplified products from the stria vascularis. Bands for β_1- and β_2-ARs are detected at the expected sizes in mRNA isolated from the stria vascularis. β_3-AR was not detected. *GAPDH* glyceraldehyde-3-phosphate dehydrogenase

expression was also confirmed, but it was rather weaker than that of β_2-ARs. Expression of β_1-ARs was much weaker than that of β_3- and β_2-ARs. No specific staining was observed for α_1-, α_{2A}- and α_{2B}-ARs. The subtypes of α_1-ARs were not tested. Intense staining was confirmed in the positive controls for each antibody (Supplemental Fig. 1), and no specific staining was observed in the negative control.

Discussion

ARs are pharmacologically classified into the following receptor types: α_1-AR (consisting of three subtypes: α_{1A}, α_{1B}, and α_{1D}); α_2-AR (consisting of α_{2A}, α_{2B} and α_{2C} subtypes), and β_1-, β_2- and β_3-AR. RT-PCR was used to analyze the mRNA expression of seven target receptor subtypes in the ES epithelia. The immunohistochemical studies using specific antibodies verified the strongest staining for β_2-ARs and moderate staining for β_3-ARs in the epithelial cells for the intermediate portion of the ES. Previous reports indicated that the intermediate portion of the ES could be the most important part that is involved in endolymph absorption (Hoshikawa et al. 1994; Dahlmann and von During 1995; Miyashita et al. 2007). Catecholamines have been reported to depress ESP through β_2-ARs, indicating that β_2-ARs may mediate their action in the ES (Mori and Uozumi 1991). The strong staining for β_2-ARs in the epithelial cells of the ES intermediate portion corresponds to the results of physiological and pharmacological analyses. These results suggest that the β_2-ARs in the intermediate portion of the ES may possibly be involved in the regulation of ES functions.

The ES epithelia contained transcripts for other ARs that were not detected in the immunohistochemical tests. The LCM samples for RT-PCR included all portions of the ES. RT-PCR results may reflect the expression of ARs in other parts of the ES besides the intermediate portion. The results could also be due to the difference in the sensitivities of the two methods. RT-PCR is more sensitive to the levels of the target mRNA than to the immunohistochemical properties. Whether these transcripts are translated into protein also remains to be ascertained. Possibly, trace amounts of these receptors may have not been present, but may not have been detected in the immunohistochemical analysis. Unfortunately, quantitative analysis of proteins, such as western blotting, is difficult because the amount of protein obtained from the ES epithelia is minute.

The β_1-ARs, but not β_2-ARs, stimulate K^+ secretion in the strial marginal cells (Wangemann et al. 2000) and vestibular dark cells (Wangemann et al. 1999). Localization of β_1-ARs in the strial marginal cells and vestibular dark cells has been confirmed by immunohistochemical analyses (Fauser et al. 2004). In contrast, the present results suggest that β_2-ARs may be predominant in the ES. The physiological significance of the difference in the predominance of β-ARs among the different parts of the endolymphatic system remains to be clarified.

It has been reported that catecholamines depress ESP without changing the endocochlear potential (Mori and Uozumi 1991) and that intravenous application of the selective β agonist isoproterenol increases the endolymphatic pressure in guinea pigs, probably through direct action on the ES (Inamoto et al. 2009). These results suggest that the ES may be the main target of catecholamines in the endolymphatic system.

In 1989 β_3-AR was cloned in humans as the third subtype of β-ARs (Emorine et al. 1989), and it has been described in white and brown adipose tissues, heart, urinary bladder, brain and blood vessels. The pharmacological roles of β_3-ARs in some tissues have been established recently. In the heart β_3-ARs have a negative inotropic action on the ventricles and are considered as new therapeutic targets for cardiovascular diseases (Gauthier et al. 2011). β_3-ARs have been found in several blood vessels in humans and other animals, including the pulmonary artery, thoracic aorta, coronary arteries and peripheral vessels, which leads to vasodilation (Coman et al. 2009). Stimulation of β_3-ARs in the adipose induces thermogenesis and causes weight loss in mice. RT-PCR results in the cochlea showed positive expression of β_1- and β_2-ARs and negative expression of β_3-AR in the stria vascularis (Wangemann et al. 2000). We reexamined the mRNA expression of the

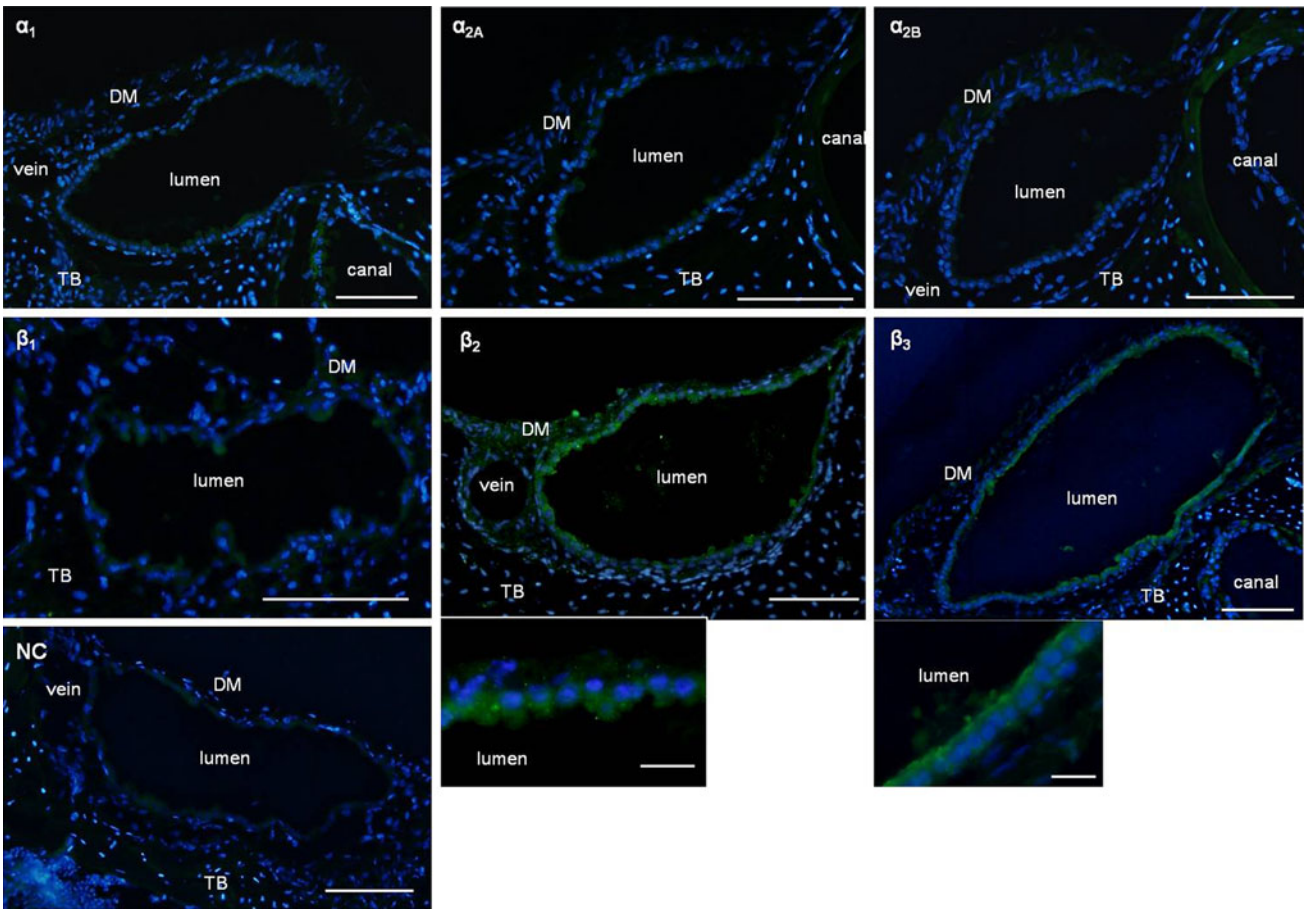

Fig. 3 Immunohistochemical analysis in the ES. The intermediate portion of the ES was stained with specific antibodies for α_1-, α_{2A}-, α_{2B}-, β_1-, β_2- and β_3-ARs. β_2- and β_3-ARs were stained with specific green in the epithelia, whereas no staining for α_1-, α_{2A}-, α_{2B}- and β_1-ARs was found. Note significant expression of β_2-ARs. Staining for β_2- and β_3-ARs is shown in high-power fields. *Scale bar* 100 μm. *Scale bar* in high-power fields = 20 μm. *NC* negative control, *lumen* lumen of the ES, *vein* vein of vestibular aqueduct, *TB* temporal bone, *DM* dura matter, *canal* semicircular canal

three β-AR subtypes in the stria vascularis using the LCM technique, which enabled the collection of total RNA with less contamination from adjacent tissues. RT-PCR analysis of the stria vascularis via LCM confirmed the expression of β_1- and β_2-ARs, with no β_3-AR expression. The presence of β_3-ARs in the ES epithelia, but not in the stria vascularis of the cochlea, suggests a specific role for β_3-ARs in the ES. Further investigations will be needed to clarify the role of β_3-ARs in the ES.

In conclusion, RT-PCR and immunohistochemistry revealed expression of β_2- and β_3-ARs in the ES epithelia for the first time. The strong immunolocalization of β_2-ARs in the intermediate portion of the ES is consistent with previous physiological and pharmacological results. The role of β_3-ARs in the ES remains to be clarified.

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