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Research report

Expression and localization of atrial natriuretic peptide and its receptors in rat spiral ganglion neurons

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ABSTRACT

Spiral ganglion neurons (SGNs) are the primary auditory neurons in the inner ear, conveying auditory information between sensory hair cells and the central nervous system. Atrial natriuretic peptide (ANP), acting through specific receptors, is a regulatory peptide required for a variety of cardiac and neuronal functions. While the localization of ANP and its receptors (NPR-A and NPR-C) in the inner ear has been widely studied, there is only limited information regarding their localization in cochlear SGNs and their regulatory roles during primary auditory neurotransmission. Here we have investigated the presence of ANP and its receptors in the cochlear spiral ganglion of the postnatal rat using immunohistochemistry, reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. ANP and its receptors are expressed in the cochlear SGNs at both the mRNA and protein level, and co-localize in the cochlear SGNs as shown by immunofluorescence. Our research provides a direct evidence for the presence and synthesis of ANP as well as its receptors in the cochlear SGNs, suggesting a possible role for ANP in modulating the neuronal functions of SGNs via its receptors.

1. Introduction

Spiral ganglion neurons (SGNs) are the primary auditory neurons in Rosenthal's canal within the modiolus of the cochlea, where they play a prominent role in the auditory system of conveying the auditory signals perceived by the sensory hair cells to the cochlear nucleus located in the brainstem (Nayagam et al., 2011; Rusznák and Szűcs, 2009). They comprise two subpopulations of neurons, the large type I neurons, representing approximately 90–95% of the afferent auditory neurons, and the small type II neurons, innervating the inner and outer hair cells respectively. Numerous factors including noise trauma, ototoxic drugs, infection, aging and genetic disorders can cause hair cell death, which leads to degeneration of SGNs, an important component of sensorineural hearing loss (Shepherd and Hardie, 2001). Understanding the expression, function and signaling interactions of the neurotransmitters, neuromodulators or other regulatory substances which affect neuronal physiology and neurotransmission in primary auditory neurons, may offer insights into the mechanisms underlying normal and pathological states of hearing, and provide important clues for effective prophylactic and therapeutic treatment for hearing impairment.

Atrial natriuretic peptide (ANP), the first member of the natriuretic peptide family with potent natriuretic, diuretic, and vasorelaxant activity, is primarily synthesized and secreted by the cardiac atria (de Bold et al., 1981). ANP exerts its actions through binding to specific high affinity receptors on the surface of target cells and it plays an important role in the regulation of cardiovascular homeostasis, maintaining blood pressure and extracellular fluid volume (Levin et al., 1998; Potter et al., 2006, 2009). ANP activates the transmembrane guanylyl cyclase (GC) natriuretic peptide receptor-A (NPR-A, or GC-A) to catalyze the synthesis of cyclic guanosine monophosphate (cGMP), which mediates the most known effects of natriuretic peptides. The natriuretic peptide clearance receptor (NPR-C) clears ANP from the circulation through receptor-mediated internalization and degradation (Levin et al., 1998; Potter et al., 2006). In addition to its well-characterized cardiac roles, recent studies have reviewed the presence and

Abbreviations: ANP, atrial natriuretic peptide; cGMP, cyclic guanosine monophosphate; CNS, central nervous system; GC, guanylyl cyclase; NPR-A, natriuretic peptide receptor-A; NPR-C, natriuretic peptide receptor-C; RT-PCR, reverse transcription-polymerase chain reaction; SGNs, spiral ganglion neurons.

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functionality of ANP in several systems, including kidney, adrenal, lung, adipose tissue, vascular smooth muscle, retina and the central nervous system (CNS) (Cao and Yang, 2008; Levin et al., 1998; Potter et al., 2006, 2009). Importantly, signaling by ANP has been linked to different neuronal functions in the CNS (Cao and Yang, 2008).

The presence of ANP and/or its receptors in the inner ear of rodents is well-documented (Chen et al., 1994; Furuta et al., 1995; Koch et al., 1992; Krause et al., 1997; Lamprecht and Meyer zum Gottesberge, 1988; Meyer zum Gottesberge et al., 1991, 1995; Meyer zum Gottesberge and Lamprecht, 1989; Qiao et al., 2011; Seebacher et al., 1999; Suzuki et al., 1998; Yoon and Anniko, 1994; Yoon and Hellstrom, 1992). These investigations suggest that ANP may play a role in the regulation of the fluid and electrolyte balance in the inner ear as a local hormone. There is some evidence that ANP receptors are localized to the cochlear modiolus of the guinea pig (Lamprecht and Meyer zum Gottesberge, 1988) and rat spiral ganglion (Furuta et al., 1995). However, no published work provides further information regarding the localization and function of ANP and its receptors in the SGNs. In our current study, we investigated the presence of ANP and its receptors (NPR-A and NPR-C) in the rat SGNs by immunohistochemistry, reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis.

2. Materials and methods

2.1. Animals and tissue preparation

All tissues were obtained from postnatal day 7 (P7) Sprague-Dawley rats (provided by the Laboratory Animal Center of the Fourth Military Medical University, Xi'an, China). The care and use of all animals in the study were carried out in accordance with the institutional guidelines of the Fourth Military Medical University. After cryoanesthesia, the rats were decapitated and the skulls were opened midsagitally. With the aid of a dissecting microscope, the cochleae were removed from the temporal bone, washed in ice-cold Hank's Balanced Salt Solution (HBSS; Invitrogen, USA) and collected for further use. In order to separate the modiolus tissues containing most of the spiral ganglia from the more peripheral tissue (the spiral ligament, stria vascularis and the organ of Corti), careful dissection was made at the border between the spiral ganglion and the spiral limbus (adapted from Whitlon et al., 2006).

2.2. RT-PCR analysis

The modiolus tissues were transferred to DNase/RNase-free microcentrifuge tubes containing RNAlater RNA Stabilization Reagent (Qiagen, USA). The total RNA was isolated from the homogenates using an RNeasy Protect Mini Kit (Qiagen) following the manufacturer's instructions. The RNA quality was determined with a spectrophotometer (BioPhotometer plus; Eppendorf, Germany). Total RNA obtained was reverse transcribed into complementary DNA (cDNA) with a PCR thermocycler (MJ Mini Personal Thermal Cycler; Bio-Rad Laboratories, USA) by using RevertAid^{TM} First Strand cDNA Synthesis Kits (Fermentas, USA). The PCR reaction consisted of 4 min at 94 °C, followed by 35 cycles of 94 °C (30 s), 55 °C (30 s), 72 °C (30 s), and ending with 10 min extension at 72 °C. The amplified products were separated on a 1% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator (Gel Doc^{TM} XR+ system; Bio-Rad). For each experiment, cardiac tissue was used as a positive control, while water was used as a negative control. All primer sequences were as follows: ANP (forward 5′-CCGGTACCGAAGATAACAGC-3′, reverse 5′-CTCCAGGAGGGTATTCACCA-3′), NPR-A (forward 5′-CCTTTCAGGCTGCCAAAAT-3′, reverse 5′-ATCCTCCACGGTGAAGTTGA-3′), NPR-C (forward 5′-TGACACCATTCGGAGAATCA-3′, reverse 5′-CATCTCCGTAAGAAGAACTGTTGA-3′), GAPDH (forward 5′-GTCGGTGTGAACGGATTTG-3, reverse 5′-CTTGCCGTGGGTAGAGTCAT-3′). All PCR experiments were performed in at least triplicate.

2.3. Western blot analysis

Total protein extract was prepared from the modiolus tissues using a lysis buffer containing 20 mM Tris–HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, and a protease inhibitor cocktail (Sigma, USA). The samples were then crushed, sonicated and centrifuged at 14,000 rpm at 4 °C for 10 min. The protein concentration in the supernatant was determined with a spectrophotometer (NanoDrop 2000c; Thermo Fisher Scientific, USA). Protein samples (50 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a polyvinylidene fluoride membrane (PVDF; Millipore, USA) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, USA). The blots were incubated for 1 h in blocking buffer containing 5% nonfat dry milk in PBS-T (0.1% Tween 20 in PBS) and then incubated overnight at 4 °C with primary antibodies (rabbit anti-ANP, goat anti-NPR-A and goat anti-NPR-C, all 1:500; Santa Cruz, USA) diluted in blocking buffer. The blots were washed in PBS-T and incubated for 2 h at room temperature with the appropriate peroxidase-conjugated secondary antibodies (1:1000, Santa Cruz), and developed using enhanced chemoluminescence reagent (Thermo Fisher Scientific). Immunoreactive bands were visualized on a chemiluminescence system (FluorChem FC2; Alpha Innotech, USA). For each experiment, a total protein extract from cardiac tissue was used as a positive control. All Western blot experiments were performed in at least triplicate.

2.4. Immunohistochemical analysis

The cochlear tissue sections were processed for immunofluorescence as follows. Briefly, the cochleae were fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.2) overnight at 4 °C, and cryoprotected in 10% sucrose for 4 h, followed by 30% sucrose overnight. All cochleae were then embedded in Tissue-Tek OCT compound (Sakura Finetek, USA) and frozen at −20 °C. The samples were sectioned into 16 μm-thick mid-modiolar cross-sections using a cryostat microtome (CM1850; Leica, Germany) and mounted on poly-L-lysine-coated slides. After washing with 0.01 M phosphate buffered saline (PBS, pH 7.4), all specimens were blocked with 5% normal donkey serum (NDS, Jackson ImmunoResearch, USA) in PBS containing 0.1% Triton X-100 for 1 h, and incubated overnight at 4 °C with polyclonal mouse anti-Tubulin β-III (TuJ1) antibody, polyclonal rabbit anti-ANP antibody, and either polyclonal goat anti-NPR-A or anti-NPR-C antibody (all 1:250; Santa Cruz) in antibody solution (1% NDS, 0.1% Triton X-100 in PBS). After washing, the samples were treated with Alexa Fluor 594-conjugated donkey anti-mouse IgG (1:800; Invitrogen), Alexa Fluor 647-conjugated donkey anti-goat IgG and DyLight 405-conjugated donkey anti-rabbit IgG (both 1:500; Jackson ImmunoResearch) in antibody solution at room temperature for 1 h. Each experiment also included a control where the primary antibody was omitted. After rinsing, the samples were mounted with Vectashield antifade mounting medium (Vector Laboratories, USA) and examined under a spectral scanning laser confocal microscope (FV1000; Olympus, Japan). All images were saved as TIFF files using Olympus confocal software FV10-ASW 1.7a and processed with Adobe Photoshop CS5 (Adobe Systems, USA) for adjustments of brightness and/or contrast.