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DEVELOPMENT OF GAP JUNCTIONAL INTERCELLULAR COMMUNICATION WITHIN THE LATERAL WALL OF THE RAT COCHLEA

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Abstract—Auditory function depends on gap junctional intercellular communication (GJIC) between fibrocytes within the cochlear spiral ligament, and basal cells and intermediate cells within stria vascularis. This communication within the lateral wall is hypothesized to support recirculation of K^+ from perilymph to the intra-strial space, and thus is essential for the high [K^+] measured within endolymph, and the generation of the endocochlear potential. In rats, the [K^+] within endolymph reaches adult levels by postnatal day 7 (P7), several days before hearing onset, suggesting that GJIC matures before auditory responses are detectable. In this study we have mapped the postnatal development of GJIC within the cochlear lateral wall, to determine the stage at which direct communication first exists between the spiral ligament and stria vascularis. Connexin 30 immunofluorescence revealed a progressive increase of gap junction plaque numbers from P0 onwards, initially in the condensing mesenchyme behind strial marginal cells, and spreading throughout the lateral wall by P7–P8. Whole-cell patch clamp experiments revealed compartmentalized intercellular dye-coupling in the lateral wall between P2 and P5. There was extensive dye-coupling throughout the fibrocyte syncytium by P7. Also, by P7 dye introduced to fibrocytes could also be detected within strial basal cells and intermediate cells. These data suggest that lateral wall function matures several days in advance of hearing onset, and provide anatomical evidence of the existence of a putative K^+ recirculation pathway within the cochlear lateral wall.

Key words: connexin, deafness, endocochlear potential, gap junctions, spiral ligament, stria vascularis.

Auditory transduction relies on the flow of K^+, from the endolymph in scala media, into sensory hair cells located in the sensory epithelium (Hibino and Kurachi, 2006; Zdebik et al., 2009). This flow is gated by mechano-sensory ion channels within the stereociliary bundles located at the apices of the hair cells, and is driven in part by the large positive extracellular potential measured within scala media, known as the endocochlear potential (EP). Both the uniquely high [K^+] of endolymph, and EP, are derived from the normal homeostatic function of the tissues within the cochlear lateral wall, namely the spiral ligament and stria vascularis (Wangemann, 2006). The spiral ligament lies immediately medial to the otic bony capsule, and consists of a strip of mesenchyme-derived fibrocytes. Based on their anatomical specializations and their expression of transporter proteins, the differentiated fibrocytes have been assigned various roles in cochlear fluid homeostasis (Spicer and Schulte, 1991, 1996; Sakaguchi et al., 1998; Furness et al., 2009). Stria vascularis directly abuts the medial face of the ligament, and is a specialized ion-transporting epithelium that encloses a complex capillary network. It consists of mesenchyme-derived basal cells, intermediate cells (melanocytes), and epithelial marginal cells (Forge and Wright, 2002). Several lines of evidence suggest that pathological changes within the lateral wall lead to inherited deafness and/or age-related hearing loss (Schuknecht and Gacek, 1993; Sakaguchi et al., 1998; Ichimiya et al., 2000; Spicer and Schulte, 2002; Rozengurt et al., 2003; Teubner et al., 2003).

Many of the cells within the lateral wall are inter-connected via gap junctions, which are constructed from connexons ("hemichannels") consisting of connexin 26 (Cx26) and/or Cx30 subunits (Ahmad et al., 2003; Forge et al., 2003a,b; Zhao et al., 2006; Liu and Zhao, 2008; Nickel and Forge, 2008; Nickel et al., 2009). Gap junction plaques are observed between the majority of fibrocyte subtypes, at the membranes between fibrocytes and strial basal cells, and at the intrastrial membranes between basal cells and intermediate cells (Forge et al., 2003a). Together, these cells are known as the "connective tissue gap junction network" (Kikuchi et al., 2000). Mutations within the genes for Cx26 (*GJB2*) and Cx30 (*GJB6*) cause inherited hearing loss in humans (Kelsell et al., 2001; Nickel and Forge, 2008), and so deficits in gap junctional intercellular communication (GJIC) within the connective tissue gap junction network may contribute to this impairment. Cx30 deficient mice have low endolymphatic [K^+] and never develop an effective EP (Teubner et al., 2003). However, in a hearing-impaired mouse model expressing a human Cx30 mutation, EP values are normal (Schutz et al., 2010).

Before the onset of hearing in rats (usually around postnatal day 12, P12), EP has been measured below +20 mV (Bosher and Warren, 1971; Rybak et al., 1992). Around P12 there is a rapid upswing of the measured value of EP, which increases to a mature level of around +90 mV by P15. Importantly, the ionic make-up of en-

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Abbreviations: bc, basal cell; cm, condensing mesenchyme; Cx26, connexin 26; Cx30, connexin 30; EP, endocochlear potential; fc, fibrocytes; GJIC, gap junctional intercellular communication; ic, intermediate cell; Ko, Kölliker's organ; lim, spiral limbus; lw, lateral wall; mc, marginal cell; oc, otic capsule; PBS, phosphate buffered saline; Rm, Reissner's membrane; sl, spiral ligament; sm, scala media; sv, stria vascularis.

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dolymph within scala media is established earlier in development. The [K^+] and [Na^+] of endolymph reach near-mature levels by P7 (Bosher and Warren, 1971). Comparable development has been observed for the mouse cochlea (Yamasaki et al., 2000). This suggests that ion transporting activity within the lateral wall matures several days in advance of hearing onset, and points to the possibility of viable GJIC within the connective tissue gap junction network during the first postnatal week. The present study was designed to examine the development of GJIC in the lateral wall of the rat cochlea, from birth until the onset of hearing. We demonstrate that there is gap junctional connectivity between undifferentiated lateral wall fibrocytes as early as P2, and between morphologically mature fibrocytes and basal/intermediate cells by P7. These observations support the hypothesis of gap junctional recirculation of K^+ via the connective tissue gap junction network, which could support the supply of K^+ to endolymph.

EXPERIMENTAL PROCEDURES

Animals

The present study was carried out on Sprague Dawley rat pups (P0–P12), reared at University College London. All procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986, and were approved by the UCL Animal Ethics Committee. Rats were deeply anesthetized with intra-peritoneal sodium pentobarbital (50 mg/kg) and decapitated.

Antibodies

The rabbit polyclonal anti-Cx30 antibody (Zymed, San Francisco, CA, USA) was used at a final concentration of 1:400. This antibody was used in some experiments as a marker of gap junction plaques. The anti-Cx30 antibody labeled large intercellular gap junction plaques in HeLa cells transfected with Cx30 cDNA, but it did not detect plaques in HeLa cells transfected with Cx26 cDNA (not shown). The rabbit polyclonal anti-Kir4.1 antibody (obtained from Alomone Labs, Jerusalem, Israel) was used at a final concentration of 1:400. In some experiments this antibody was used to delineate the intermediate cells within stria vascularis, as shown elsewhere (Ando and Takeuchi, 1999; Jagger et al., 2010).

Immunofluorescence

Bullae tympanicae were removed and the otic capsule exposed. A small hole was made in the apex of the otic capsule, and the whole cochlea was perfused with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). Cochleae were fixed in PFA for 30 min at room temperature and then washed several times in PBS. For vibratome slices, the otic capsules were mounted on a vibratome block using cyanoacrylate adhesive and sectioned at 100–200 μm. For transverse cryosections, cochleae were cryoprotected in a 30% sucrose-PBS solution at 4 °C overnight, and embedded the following day in 1% low-temperature gelling agarose (type VII, Sigma) made up in an 18% sucrose-PBS solution. Agarose blocks containing the cochleae were mounted onto cryostat chucks with Tissue Tek embedding medium (Miles, Inc., Elkhart, IN, USA) and snap frozen in liquid nitrogen. Sections were cut at 15–20 μm thickness at −25 °C on a cryostat, mounted on polylysine-coated slides, dried at room temperature for 1 h, and stored at −80 °C until used. Differential interference contrast micrographs were taken using a digital camera mounted on an upright microscope (Axioplan 2 Imaging; Carl Zeiss MicroImaging, Jena, Germany). Vibratome slices or cryosections were permeabilized and blocked (0.1% Triton-X 100 with 10% normal goat serum in PBS) for 30 min at room temperature, and then incubated in primary antibodies overnight at 4 °C. In controls primary antibodies were omitted. Following several PBS washes, slices were incubated in Alexa-Fluor tagged secondary antibodies (Invitrogen, Paisley, UK) in the dark for 60 min at room temperature. Slides were cover-slipped and mounted using Vectashield with DAPI (Vector Labs, Peterborough, UK). Imaging was carried out using a laser scanning confocal microscope (LSM510; Carl Zeiss MicroImaging) as described elsewhere (Jagger and Forge, 2006). Micrographs were converted to TIFF format, and adjusted for optimal contrast and brightness.

Whole-cell recording and dye injections

Dissection was performed in cold artificial perilymph (in mM: 140 NaCl, 4 KCl, 1 $MgCl_2$, 1.3 $CaCl_2$, 10 HEPES, and 5 glucose; pH adjusted to 7.3 with NaOH). All chemicals were obtained from Sigma unless stated. Live cochlear slice preparations were obtained as described elsewhere (Jagger and Forge, 2006; Furness et al., 2009). Slices cut at 200 μm thickness were stored in artificial perilymph on ice until used. For recordings, slices were placed in a recording chamber (volume 400 μl) mounted on an upright microscope (E600FN, Nikon, Tokyo, Japan) and were super-fused with artificial perilymph (1 ml/min). Slices were held beneath short lengths of platinum wire to prevent movement. Experiments were conducted at room temperature (20–24 °C). Patch clamp recordings were performed under infrared differential interference contrast (IR-DIC) video-microscopy, using a CCD video camera and IR-DIC optics mounted on the microscope. Dyes were injected into cells during 10-min whole-cell recordings. Recordings were performed using a patch clamp amplifier (Axopatch 200B; Axon Instruments, Foster City, CA, USA) and a Digidata board (Axon Instruments) under the control of computer software (pClamp version 8; Axon Instruments). Patch pipettes were fabricated on a vertical puller (Narishige) from capillary glass (GC120TF-10; Harvard Apparatus, Edenbridge, UK). Pipettes were filled with a KCl-based solution (in mM: 140 KCl, 10 NaCl, 2 $MgCl_2$, 5 HEPES, 0.5 EGTA, 3 Na_2ATP, and 5 glucose, pH adjusted to 7.3 with KOH). In all experiments this solution was supplemented with 0.2% neurobiotin (molecular weight, 287 Da; charge, +1; Vector Labs), and in some experiments 0.2% Fluorescein Dextran (10 kDa; anionic; Invitrogen) was added. Pipette solutions were filtered at 0.2 μm and centrifuged to remove small insoluble particles. Pipettes had an access resistance of 2–3 MΩ, measured in artificial perilymph. At the termination of recordings, slices were fixed immediately in 4% PFA for 20 min at room temperature. Following fixation, slices were prepared for immunofluorescence as described above. To counter-stain gap junction plaques, some slices were incubated in the anti-Cx30 antibody, and then in the appropriate secondary antibody as described above. To visualize neurobiotin, Alexa555-Fluor tagged streptavidin (1:1000; Invitrogen) was added with the secondary antibody. Slides were cover-slipped using Vectashield with DAPI (Vector Labs), and confocal imaging was carried out as described above.

RESULTS

Development of gap junction plaques within the postnatal cochlear lateral wall

The rat cochlea undergoes extensive morphological development between birth and the onset of hearing (around P12). At P0, hair cells and their supporting cells were clearly distinguishable within the sensory epithelium (Fig. 1A, arrow). Within the lateral wall, marginal cells formed a