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SYNAPTIC ORGANIZATION IN COCHLEAR INNER HAIR CELLS DEFICIENT FOR THE $Ca_V1.3$ (α1D) SUBUNIT OF L-TYPE Ca^{2+} CHANNELS

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Abstract—Cochlear inner hair cells (IHCs) release neurotransmitter onto afferent auditory nerve fibers in response to sound stimulation. Normal development and function of inner hair cells require the expression of α subunit 1.3 forming L-type voltage-gated Ca^{2+} channel ($Ca_V1.3$).

Here, we used immunohistochemistry and reverse transcription-polymerase chain reaction to study the synaptic organization and expression of large conductance Ca^{2+}-activated potassium channels in IHCs of mice lacking the $Ca_V1.3$ Ca^{2+} channel ($Ca_V1.3^{-/-}$). Despite the near complete block of evoked afferent synaptic transmission, hair cell ribbon synapses were formed and remained preserved for at least 4 weeks after birth. Moreover, these "silent" afferent synapses held major components of the synaptic machinery such as Bassoon, Piccolo, and CSP. Hence, the block of exocytosis might be solely attributed to the lack of Ca^{2+} influx through $Ca_V1.3$ channels. Later on, $Ca_V1.3$ deficient IHCs subsequently lost their afferent synapses. This was probably due to a secondary degeneration of the postsynaptic spiral ganglion neurons.

In line with a prolonged efferent synaptic transmission onto $Ca_V1.3$ deficient IHCs, which normally ceases around onset of hearing, we found juxtaposed immunoreactive spots of efferent presynaptic synaptophysin and postsynaptic (IHCs) small conductance Ca^{2+}-activated potassium channels (SK channels) up to six weeks after birth. Finally, we show a substantial reduction of mRNA for the α subunit of the large conductance Ca^{2+}-activated potassium channel (BK) in the apical cochlea, suggesting a reduced transcription of its gene in $Ca_V1.3$ deficient IHCs. $Ca_V1.3$ deficient IHCs lacked the apical spot-like immunoreactivity of clustered BK channels, which normally contribute to the temporal precision of hair cell afferent synaptic transmission.

In summary, these data indicate that the $Ca_V1.3$ channels are crucially involved in regulation of the expression of BK and SK channels. $Ca_V1.3$ channels seem not to be essential for ribbon synapse formation, but are required for the maintenance of ribbon synapses and spiral ganglion neurons.

Key words: calcium channel, hair cell, ribbon synapse, synaptogenesis, efferent synapse, BK channel.

Inner hair cells (IHCs) are the genuine sensory cells of the mammalian cochlea that transmit mechanical stimuli into neuronal signals (Fuchs et al., 2003, Nouvian et al., 2006). About 95% of the primary auditory neurons (type I spiral ganglion neurons, SGNs) receive their input from IHCs using AMPA type glutamate receptors (GluRs) (Matsubara et al., 1996; Glowatzki and Fuchs, 2002). Sound-evoked gating of the IHC's mechanotransducer channels causes a depolarizing receptor potential, which in turn activates basolateral voltage-gated Ca^{2+} and K^+ channels. In contrast to neuronal presynaptic terminals, IHC afferent synapses mainly contain L-type Ca^{2+}-channels, ~90% of which are α subunit 1.3 forming L-type voltage-gated Ca^{2+} channels ($Ca_V1.3$) (Platzer et al., 2000; Brandt et al., 2003; Dou et al., 2004). These channels cluster at the active zones, where release of each fusion-competent synaptic vesicle is probably under Ca^{2+} nanodomain control exerted by one or few Ca^{2+} channels (Brandt et al., 2005).

Consequently, depolarization-evoked exocytosis is nearly abolished in $Ca_V1.3$ deficient IHCs (Brandt et al., 2003). However, robust exocytosis, as assayed by membrane capacitance measurements, could still be evoked when bypassing the lacking Ca^{2+} influx by intracellular release of Ca^{2+} using flash photolysis of caged Ca^{2+} (Brandt et al., 2003). It remained unclear, however, whether the block of depolarization-induced exocytosis in $Ca_V1.3$ deficient IHCs and the deafness included a more general defect of synaptic organization. In fact, a reduction of synaptic ribbons had been suggested for $Ca_V1.3$ deficient IHCs in a previous electron microscopy study (Glueckert et al., 2003). Hence, it remained possible that the preserved exocytosis evoked by Ca^{2+} uncaging was unrelated to synaptic transmission at ribbon synapses. In order to investigate ribbon synapse formation and to more quantitatively study the molecular anatomy of the afferent hair cell synapses in $Ca_V1.3$-deficient IHCs, we performed immunohistochemistry for afferent synaptic markers at several developmental stages. This method has a high detection efficiency for ribbon-containing synapses, which are recognized as juxtaposed spots of presynaptic RIBEYE (ribbon marker, Schmitz et al., 2000) and postsynaptic ionotropic GluR immunofluorescence in three dimensional (3D) reconstructions of the organ of Corti (Khimich et al.,

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Abbreviations: BK, large conductance calcium-activated potassium channel; $Ca_V1.3$, α subunit 1.3 forming L-type voltage-gated Ca^{2+} channels; cDNA, complementary DNA; CSP, cysteine string protein; GluR, glutamate receptor; GSDB, goat serum dilution buffer; IHC, inner hair cell; *KCNMA1*, the large conductance calcium-activated potassium channel, subfamily M, α subunit 1; *Kcnma1*, mouse gene coding KCNMA1; NF-200, neurofilament of 200 kDa; PFA, paraformaldehyde; p8, postnatal day 8; RT-PCR, reverse transcription-polymerase chain reaction; SK, small conductance Ca^{2+} activated K^+ channel; TBP, TATA-binding protein; 2D, two dimensional; 3D, three dimensional.

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2005). It also enables one to collect a large sample of synapses with moderate effort. In addition to RIBEYE we investigated the presence of key synaptic proteins Bassoon, Piccolo and cysteine string protein (CSP) in the functionally "silent" $Ca_V1.3$ deficient IHCs.

Cochlear degeneration is the common final condition of many inner ear pathologies, including inherited cochlear dysfunction as well as toxic or noise damage to the cochlea. Eventually the failure or degeneration of the sensory cells leads to secondary loss of SGNs (Spoendlin, 1984). This has been mainly attributed to the loss of trophic support for SGNs by IHCs and supporting cells (Rubel and Fritzsch, 2002; Stankovic et al., 2004). The $Ca_V1.3$ mutant provided an excellent paradigm to specifically study the consequences of an IHC synaptic failure on the ascending auditory pathway, as IHCs are initially preserved (Platzer et al., 2000; Dou et al., 2004). Indeed, a loss of type I SGNs was observed in these mice (Glueckert et al., 2003). However, this previous study did not quantify the afferent fibers projecting to the "silent" IHCs, which may be reduced well before loss of the neuronal somata. Therefore, in addition to observing postsynaptic GluR clusters, we studied the abundance of afferent SGN fibers projecting toward $Ca_V1.3$-deficient IHCs.

Besides their impact on synaptic function, $Ca_V1.3$ channels seem to be essential for regulation of hair cell development. For example, the efferent synaptic control by fibers of the medial olivocochlear bundle that is normally lost after the onset of hearing (Zuo et al., 1999; Glowatzki and Fuchs, 2000; Simmons, 2002; Brandt et al., 2003) seems to be maintained for several weeks in $Ca_V1.3^{-/-}$ mice (Brandt et al., 2003). At these cholinergic synapses unconventional ACh receptors formed from $\alpha9$ and $\alpha10$ subunits (Plazas et al., 2005) mediate a non-selective cation influx into IHCs. The incoming Ca^{2+} causes activation of small conductance Ca^{2+}-activated potassium channels (SK channels; Glowatzki and Fuchs, 2000; Oliver et al., 2000). The SK current results in a hyperpolarization of IHCs, which may interfere with the generation of Ca^{2+} action potentials (Glowatzki and Fuchs, 2000). While outer hair cells maintain efferent cholinergic inhibition throughout their life, IHCs loose the efferent inhibition during the second postnatal week (Katz et al., 2004). In order to explore the morphological basis of our previous physiological finding of prolonged efferent control of IHCs, we used immunohistochemistry to identify efferent hair cell synapses as juxtaposed spots of presynaptic synaptophysin and postsynaptic SK channels in control and $Ca_V1.3$ deficient IHCs at various developmental stages.

Furthermore, $Ca_V1.3$ channels are essential for the acquisition of fast outward currents mediated by large conductance Ca^{2+} activated potassium channels (BK channels) around the onset of hearing, which is another hallmark of normal IHC development (Kros et al., 1998). BK currents could not be observed in $Ca_V1.3$ deficient IHCs up to the fifth postnatal week (Brandt et al., 2003). It remained unclear, however, whether this was due to failure of transcription of the *Kcnma1* gene coding for the pore-forming α subunit of the BK channel, translation or targeting of normally synthesized protein to the plasma membrane. Here, we performed reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry in order to address this question.

EXPERIMENTAL PROCEDURES

Solutions

Hepes–Hank's solution for explanting the organ of Corti contained (in mM): 141 NaCl, 5.4 KCl, 1 $MgCl_2$, 0.5 $MgSO_4$, 6 L-glutamine, 7 glucose, and 10 NaOH–Hepes, pH 7.2. 120 mM phosphate buffer contained: 100 mM Na_2HPO_4, 20 mM NaH_2PO_4.

GSDB (goat serum dilution buffer) contained: 16% normal goat serum, 450 mM NaCl, 0.3% Triton X-100, 20 mM phosphate buffer, pH 7.4. Fixative contained either 4% paraformaldehyde (PFA) in 120 mM phosphate buffer, 99.9% ethanol or 99.9% methanol. Phosphate buffer saline contained (in mM): 140 NaCl, 2.7 KCl, 8 Na_2HPO_4, and 1.5 KH_2PO_4, pH 7.4. Wash buffer contained: 450 mM NaCl, 20 mM phosphate buffer, 0.3% Triton X-100.

Antibodies

The following antibodies were used: mouse anti-Bassoon Sap7f (diluted 1:1000–1:500, generated against amino acids 756–1001 of the Bassoon protein, gift of E. Gundelfinger, Magdeburg, Germany), guinea-pig anti-Piccolo [1:1000, generated against amino acids 2172–2361 of Piccolo protein (Fenster et al., 2000), gift of E. Gundelfinger, Magdeburg, Germany], Anti-CSP (1:400; Chemicon, Temecula, CA, USA), Anti-CtBP2 mouse IgG1 (1:100–200; BD Biosciences, Pharmigen, San Diego, CA, USA; recognizing the B-domain of RIBEYE and the transcriptional co-repressor CtBP2 [c-terminal binding protein 2, hence both ribbons and nuclei are stained]; ribbon-staining overlapping with RIBEYE A-domain staining in IHCs, Khimich et al., 2005), rabbit anti-GluRs 2/3 affinity purified polyclonal antibody (1:300–1:1000; Chemicon), mouse anti-parvalbumin235 monoclonal antibody (1:500; against parvalbumin; Swant, Bellinzona, Switzerland), rabbit anti-parvalbumin28 (1:500, Swant), rabbit anti-BK_{Ca} channel (1:300; Alomone Laboratories, Jerusalem, Israel; recognizing the intracellular C-terminus of KCNMA1), mouse monoclonal anti-neurofilament-200 (NF-200, 1:150–200, Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-potassium channel SK2 (1:200; generated against amino acids 542–559 of potassium SK2 channel; Sigma-Aldrich), mouse monoclonal anti-Synaptophysin (1:400, Synaptic Systems, Goettingen, Germany), Alexa Fluor 568 (1:200, goat anti-rabbit IgG, Molecular Probes, Eugene, OR, USA), Alexa Fluor 568 (1:200, goat anti-mouse IgG, Molecular Probes), Alexa Fluor 488 (1:200, goat anti-mouse IgG, Molecular Probes), Alexa Fluor 488 (1:200, goat anti-guinea-pig IgG, Molecular Probes).

For Fig. 4C–D Hoechst 34850 (1:1000, nucleic acid stain, Molecular Probes) was used to stain the nuclei in the organ of Corti.

Preparation of the organ of Corti

$Ca_V1.3^{-/-}$ mice (Platzer et al., 2000; kind gift of J. Striessnig, Innsbruck, Austria; mice were backcrossed for at least five generations into a C57BL/6N genetic background) or wild-type mice (wt) (C57BL/6N) of the specified age were killed by decapitation, according to national ethical guidelines. Animal handling followed national ethical guidelines. The number and suffering of animals were kept as low as possible. The skull was then sagittally cut into two parts that were placed into Petri dishes with ice-cooled Hepes–Hank's solution. Under the dissecting microscope, the brain was removed and the bulla was opened to expose the cochlea. The cochlea's bony envelope was carefully opened and the apical coil of the organ of Corti was removed with fine forceps.