Molecular Identity and Functional Properties of a Novel T-Type Ca\(^{2+}\) Channel Cloned From the Sensory Epithelia of the Mouse Inner Ear

Liping Nie,1,2,* Jun Zhu,1,2,3,* Michael Anne Gratton,4 Amy Liao,4 Karen J. Mu,1,2 Wolfgang Nonner,5 Guy P. Richardson,6 and Ebenezer N. Yamoah1,2

1Center for Neuroscience and 2Program in Communication Science, University of California Davis, Davis, California; 3Department of Nephrology, Xiying Hospital, The Fourth Military Medical University, Xi’an, China; 4Department of Otorhinolaryngology, University of Pennsylvania, Philadelphia, Pennsylvania; 5Department of Physiology and Biophysics, University of Miami School of Medicine, Miami, Florida; and 6School of Biological Sciences, University of Sussex, Brighton, United Kingdom

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Nie L, Zhu J, Gratton MA, Liao A, Mu KJ, Nonner W, Richardson GP, Yamoah EN. Molecular identity and functional properties of a novel T-type Ca\(^{2+}\) channel cloned from the sensory epithelia of the mouse inner ear. J Neurophysiol 100: 2287–2299, 2008. First published August 27, 2008; doi:10.1152/jn.90707.2008. The molecular identity of non-Cav1.3 channels in auditory and vestibular hair cells has remained obscure, yet the evidence in support of their roles to promote diverse Ca\(^{2+}\)-dependent functions is indisputable. Recently, a transient Cav3.1 channel current that serves as a functional signature for the development and regeneration of hair cells has been identified in the chicken basilar papilla. The Cav3.1 current promotes spontaneous activity of the developing hair cell, which may be essential for synapse formation. Here, we have isolated and sequenced the full-length complementary DNA of a distinct isoform of Cav3.1 in the mouse inner ear. The channel is derived from alternative splicing of exon14, exon25A, exon34, and exon35. Functional expression of the channel in Xenopus oocytes yielded Ca\(^{2+}\) currents, which have a permeation phenotype consistent with T-type channels. However, unlike most multion channel, the T-type channel does not exhibit the anomalous mole fraction effect, possibly reflecting comparable permeation properties of divalent cations. The Cav3.1 channel was expressed in sensory and nonsensory epithelia of the inner ear. Moreover, there are profound changes in the expression levels during development. The differential expression of the channel during development and the pharmacology of the inner ear Cav3.1 channel may have contributed to the difficulties associated with identification of the non-Cav1.3 currents.

INTRODUCTION

Voltage-gated Ca\(^{2+}\) (Ca\(_v\)) channels (VGCCs) have evolved into highly diverse structures with respect to their functional properties, subcellular assembly, and regulation to confer multiple functions in the nervous system. Using molecular biological and functional assays, Ca\(_v\),1.3 currents have been identified in auditory and vestibular hair cells of different species such as chick, frog, mouse, and human (Art and Fettiplace 1987; Bao et al. 2003; Fuchs et al. 1990; Hudspeth and Lewis 1988; Kollmar et al. 1997; Michna et al. 2003; Rodriguez-Contreras and Yamoah 2001, 2003; Rodriguez-Contreras et al. 2002; Schnee and Ricci 2003; Zidanic and Fuchs 1995). The role of the Ca\(_v\),1.3 in hair cells is underpinned by the evidence that Ca\(_v\),1.3-deficient (Ca\(_v\),1.3\(^{-/-}\)) mice are deaf, demonstrating a significant reduction in whole cell Ca\(^{2+}\) current in inner hair cells (IHCs) (Dou et al. 2004; Platzer et al. 2000). However, a residual Ca\(^{2+}\) current remains in IHCs and outer hair cells (OHCs) of Ca\(_v\),1.3\(^{-/-}\) mice. These mice no apparent vestibular disorder was observed (Dou et al. 2004), suggesting that cochlear and vestibular hair cells may express multiple VGCCs. Moreover, data from Ca\(_v\),1.3\(^{-/-}\) mice strongly suggest that non-Cav3.1 channel currents suffice to maintain normal cellular morphology and function in vestibular sensory organs (Dou et al. 2004). Recent reports have demonstrated the fleeting expression of T-type currents in the chicken basilar papilla, during hair-cell development and regeneration (Levic et al. 2007). These findings are essential to uncover the mechanism of hair-cell regeneration and restoration of hearing. Thus identification of the diverse Ca\(^{2+}\) channel subtypes in hair cells is key to our understanding of the multiple Ca\(^{2+}\)-dependent functions.

Previous studies have used pharmacological, immunohistochemical, and functional techniques to identify non-Cav1,1.3 channel in hair cells. These studies have suggested the presence of Ca\(_v\),2.2 channels in hair cells of the frog saccule (Rodriguez-Contreras and Yamoah 2001; Su et al. 1995). Similarly, the Ca\(_v\),2.3 channels have been identified in hair cells within the vestibule (Martini et al. 2000). Furthermore, earlier recordings from mammalian vestibular hair cells showed that a portion of the Ca\(_v\),2.3 channel current was derived from a transient low-voltage-activated (LVA) current, unlike a typical Ca\(_v\),2.x current (Rennie and Ashmore 1991). Additionally, a recent report has identified T-type currents in rat OHCs (Inagaki et al. 2008). This observation, together with another recent report of a transient Ca\(^{2+}\) current in the chicken basilar papilla (albeit transitory), suggests that Ca\(_v\),3.x channels are possible candidates of a hair-cell non-Cav1,1.3 channel subtype (Levic et al. 2007). The role of T-type channels in tonically active hair cells remains undetermined. Even more perplexing is the fact that the non-Cav3.1 currents in hair cells do not always exhibit exemplary fast inactivation of T-type currents (see Levic et al. 2007). Here, we have cloned a distinct inner ear Ca\(_v\),3.1 channel, derived from alternative splicing of the mouse brain Ca\(_v\),3.1 isoform. Functional expression of the channel yielded currents with the characteristic T-type current profile. Unlike
most multi-ion channels, however, the T-type channel did not exhibit an anomalous mole fraction effect (AMFE) and the underlying mechanisms for its aberrant property were discussed. Moreover, we have demonstrated the differential expression of the Ca$_{3.1}$ channel, during development in the sensory and nonsensory epithelia of the mouse cochlea and vestibule.

**METHODS**

**Polymerase chain reaction (PCR) analysis of Ca$_{3.1}$ channels in the inner ear**

Mice were housed and killed using approved protocols by the University of California, Davis, IACUC committee on Animal Research Services. Total RNA was isolated from microdissected mouse cochlear and utricular tissues, using the RNeasy Mini kit (Qiagen). The first strand of complementary (c)DNA was synthesized from 1 µg of total RNA using Arrayscript reverse transcriptase (Ambion) and random primers by incubating initially at room temperature for 5 min and then at 42°C for 2 h. The reaction was terminated by heating at 70°C for 15 min. Finally, the cDNAs were treated with RNaseH for 20 min at 37°C before further use. PCR was performed using primer pairs corresponding to known mouse Ca$^{2+}$ channel sequences as subsequently indicated. For Ca$_{1.3}$, sense: GCT CAA TGG CAG TGT GTG TC and antisense: GTC TGG CTC CTC CTC GTC ACTG (239–258 and 350–332 of AK018426, respectively), and for Ca$_{3.3}$ sense: AGG CCA AGA GTT CCT TGG AC and antisense: AGC CGA CTT GCC ATT ACAG (3611–3630 and 3705–3723 of NM-009783.1, respectively). The PCR reaction was initiated by denaturation at 94°C for 1 min, followed by 50 cycles of amplification consisting of 30 s at 94°C, 30 s at 52°C, and 45 s at 72°C. The resultant PCR product was separated on a 2% agarose gel and purified using a Qiagen gel extraction column (Qiagen). The purified PCR products were sequenced to elucidate the sequence identities.

**Molecular cloning of full-length Ca$_{3.1}$ channels**

A mouse cochlea AZAP-cDNA library of DBA mice was screened using multiple probes. The probes were PCR-amplified fragments; probes P1, P2, P3, and P4 corresponding to the regions of nucleotides 32–951, 2276–3010, 3248–3942, and 6165–6890 for the mouse Ca$_{3.1}$ cDNA (NM_009783.1), respectively. The probes were labeled by random priming with digoxigenin-UTP and approximately 1.6 million individual clones of the cDNA library were screened following the manufacturer’s instructions (Roche Molecular Biochemicals, Indianapolis, IN). Sequences of clones obtained from the cDNA library were determined by double-strand sequencing. For expression studies, the entire open reading frame of the Ca$_{3.1}$ channel was cloned into a pNL-R-XV vector, in which the channel was flanked by the 5′- and 3′-untranslated regions of a Xenopus ß-globin gene (Nie et al. 2004). From the resulting expression plasmid, cRNAs of the Ca$_{3.1}$ channel were transcribed in vitro using T7 RNA polymerase and injected into stage V–VI oocytes as described previously (Nie et al. 2004).

**Immunohistochemistry**

A rabbit polyclonal antibody for Ca$_{3.1}$ (Alomone Labs, Jerusalem, Israel) generated against the cytoplasmic N-terminus of the T-type Ca$^{2+}$ channel Ca$_{3.1}$ was used. Embryos were harvested from timed pregnant 129Sv females at E12, E15, and E18. The embryo (E12) or whole heads (E15 and E18) were fixed for 2 h at room temperature in 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Inner ear tissues were also harvested from postnatal day 0 (P0), P3, P6, P8, P12, P14, P18, P21, and adult 129Sv mice. At least three animals were examined for each time point. For P0 mice, entire heads were immersed in 4% paraformaldehyde for 2 h at room temperature. For the remainder of the ages, the sedated (Avertin [2,2,2-tribromethanol], 300 µg/gm body weight, administered intraperitoneally) mice were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PB. The temporal bones were isolated and the cochlea was perfused via the round window, then immersed in the fixative for 60 min. Following fixation the cochleae were decalcified (0.12 M EDTA, pH 7.0, 24 h, 23°C), dehydrated in a graded ethanol series, embedded in paraffin (Paraplast), and sectioned (5 µm) in the mid-modiolar plane. Sections were deparaffinized, rehydrated, and equilibrated in PBS with 5% nonimune normal goat serum (NGS, Vector Laboratories, Burlingame, CA) for 30 min and reacted (1:250) overnight at 4°C with anti-Ca$_{3.1}$ primary antibody. The PBS-rinsed sections were incubated (30 min, 23°C) in biotinylated goat anti-rabbit IgG (1:200) then rinsed and treated with Vectastain ABC reagent (Vector Labs) for 30 min. Sites of bound primary antibody were visualized by development in a 3,3′-diaminobenzidine (DAB)-H$_2$O$_2$ substrate medium (Fast DAB tablets; Sigma Chemical, St. Louis, MO). Images were captured using an Olympus BH-2 microscope fitted with a SPOT RT-KE CCD camera and image-analysis software (Diagnostic Instruments, Sterling Heights, MI). Final figures were assembled using Adobe Photoshop and Illustrator software (Adobe Systems, San Jose, CA).

**Electrophysiological recordings**

Two-electrode voltage-clamp experiments were carried out with the oocyte clamp amplifier (OC-725C; Warner Instruments, Hamden, CT). The microelectrodes were filled with 3 M KCl. Oocytes were bathed in a solution that contained (in mM) 40 NaCl, 56 N-methyl-D-glucamine (NMG), 2 KC1, 2–65 BaCl$_2$, 5 HEPES (N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]), and 1 niflumic acid to block endogenous chloride currents in oocytes (pH adjusted to 7.4 with NaOH). To maintain tonicity of the external solution (280 mosmol), the concentration of NaCl/NMG was adjusted accordingly, as the BaCl$_2$/CaCl$_2$/SrCl$_2$ concentration was increased from 2 to 65 mM. The current was activated using different voltage-clamp protocols as described in RESULTS. Liquid junction potentials were measured and corrected as described previously (Rodriguez-Contreras et al. 2002).

Standard patch-clamp recording techniques were used to record single-channel currents from Xenopus oocytes, which expressed a robust current (2–3 nA). An Axopatch 200B amplifier was used (Molecular Devices, Union City, CA). The amplitude histogram at a given test potential was generated. Leak-subtracted current recordings were idealized with a half-height criterion. Patch electrodes contained (in mM): 70 NMG-Cl, 40 KC1, 20 BaCl$_2$/CaCl$_2$, 5 HEPES, and 10 N-glucose, pH 7.4 (KOH). The bath solution contained (in mM): 140 KCl, 4 NaCl, 1 CaCl$_2$, 0.5 MgCl$_2$, 5 HEPES, and 10 N-glucose, pH 7.4 (KOH). The Q-software was used for single-channel analysis, as described previously (Rodriguez-Contreras and Yamoah 2001). Reagents were purchased from Sigma Chemical, unless specified otherwise.

**RESULTS**

**A new isoform of the mouse Ca$_{3.1}$ in the inner ear**

The expression of non-Ca$_{1.3}$ channels in the mouse inner ear was first examined by RT-PCR using Ca$_{3.1}$ channel-specific primers. Messenger RNA of Ca$_{3.1}$ channels was detected in both the cochlea and utricle of CBA mice (Fig. 1A). However, the levels of Ca$_{3.1}$ messenger RNA were qualita-
tively low compared with that of Cav1.3 channels. Motivated by this evidence, together with the existence of a transient LVA Ca\(^{2+}\) current in mammalian hair cells (Inagaki et al. 2008; Rennie and Ashmore 1991) and recent evidence demonstrating the presence of T-type Ca\(^{2+}\) currents in the developing and regenerating hair cells, we cloned a full-length Cav3.1 channel from the mouse cochlea. Using a cDNA library, we used multiple probes, which correspond to highly conserved regions of the known mouse Cav3.1 cDNA. In all, 25 clones were obtained from library screening and 6 of them were fully sequenced. Moreover, the remaining 19 were sequenced and found to be redundant. The full-length cDNA sequence of the inner ear Cav3.1 channel was determined from the assembly of overlapping clones. In addition, we performed long-range PCR to confirm the right connection of different clones. As a result, the Cav3.1 cDNA obtained from the mouse inner ear was 7281 base pairs in length (Accession number DQ317412), derived from alternative splicing that excluded exon14, exon25A, exon34, and exon35 (Fig. 1, B–D). This splice variant of mouse Cav3.1 has the same exon utilization as that of the human Ca\(^{3.1}\) isoform 11 (GenBank accession number NM_198382.1). Furthermore, the splice variant had
not been identified in mice. The splice variant encodes a putative protein of 2265 amino acids (aas) with a typical T-type channel structure: four functional domains of six transmembrane segments and a pore region. The Ca$_{3.1}$ isoform shares 94% sequence similarity with its human counterpart. As in many other cases, the highest sequence similarities appear at the transmembrane domains and the pore-forming regions (~98%), whereas the lowest was at the intracellular N- and C-termini (~84%). The functional impact of alternative splicing on the property of other isoforms of Ca$_{3.1}$ channels has been reported previously (Chemin et al. 2002a). Thus we examined the properties of the newly identified Ca$_{3.1}$ channels in the inner ear.

**Distinct properties of inner ear Ca$_{3.1}$ channel**

The inner ear Ca$_{3.1}$ channel was subcloned into the pNLRI-XV vector and expressed in *Xenopus* oocytes. Features of the Ca$_{3.1}$ channel current were determined using a two-electrode voltage-clamp approach (Fig. 2). In contrast to saline-injected oocytes, oocytes injected with about 25 nM Ca$_{3.1}$ mRNA yielded inward Ba$_{2+}$ currents (Fig. 2A). The Ba$_{2+}$ current of the inner ear Ca$_{3.1}$ channel showed voltage-dependent decay and the magnitude of the current increased as the external Ba$_{2+}$ concentration was raised (Fig. 2, A and B). Moreover, the current appeared to saturate at Ba$_{2+}$ concentrations >30 mM, which was also the case for Ca$_{2+}$ and Sr$_{2+}$ currents (Fig. 2, B and C). Data on the relations between current magnitudes and external divalent cation concentrations were fitted with a Langmuir isotherm. The estimated apparent $K_D$ values were markedly similar (Fig. 2C), as compared with the reported $K_D$ of Ca$_{1.3}$ in hair cells and chick ciliary ganglion neurons (Church and Stanley 1996; Rodriguez-Contreras et al. 2002). It should be noted that physiological Ca$_{2+}$ concentration (1–2 mM) lies within the most sensitive range of the saturation curve (Fig. 2C), suggesting that the channel operates at the optimal concentration of Ca$_{2+}$. The shift in the peak voltage and voltage-dependent activation of the current, as seen in the current–voltage (I–V) relation, was consistent with surface charge screening effects resulting from increased divalent cations (Hagiwara and Ohmori 1982; Hagiwara and Takahashi 1967; Rodriguez-Contreras and Yamoah 2003; Zhou and Jones 1995). These results were in accord with previous reports of T-type Ca$_{2+}$ currents (Huguenard et al. 1996; Huguenard et al. 1993; Perez-Reyes 1998, 2003).

Although the apparent affinity of the inner ear Ca$_{3.1}$ was similar for the three divalent cations (Ba$_{2+}$/Ca$_{2+}$/Sr$_{2+}$), the magnitude of the currents carried by Ba$_{2+}$, Ca$_{2+}$ (Fig. 3A), and Sr$_{2+}$ (Fig. 3B) were dissimilar, as shown in the summary data of the I–V relations. Invariably, the order of current sizes was Sr$_{2+}$ > Ca$_{2+}$ > Ba$_{2+}$). In contrast, findings that are in stark contrast to the permeation properties of divalent cations in Ca$_{1.3}$ follow the order Ba$_{2+}$ > Sr$_{2+}$ > Ca$_{2+}$ (Hagiwara and Ohmori 1982; Hille 2001). The half-maximal activation voltages and the slope factors of the steady-state activation curves for Ba$_{2+}$, Ca$_{2+}$, and Sr$_{2+}$ currents were ~33.2 ± 0.6, −35.5 ± 0.6, and −38.5 ± 0.6 mV; and 6.4 ± 0.5, 6.3 ± 0.6, and 5.6 ± 0.5 mV (n = 17), respectively (Fig. 3C). Using a standard steady-state inactivation protocol, a 500-ms conditioning pulse was applied to different activation potentials between −80 and 0 mV, which was then followed by a deactivation gap (2 ms) at the holding potential (−110 mV) and a test pulse to −20 mV. Current traces similar to the one shown in the inset (Fig. 3D) were used to plot the curves. Ba$_{2+}$, Ca$_{2+}$, and Sr$_{2+}$ currents were half-inactivated at −52.1 ± 0.6, −53.8 ± 0.6, and −56.8 ± 0.5 mV; and the curves had slope factors (in mV) of 5.4 ± 0.5, 4.7 ± 0.4, and 5.5 ± 0.5 (n = 14), respectively.

**FIG. 2.** Inner ear Ca$_{3.1}$ channel currents. A: examples of inner ear Ca$_{3.1}$ currents expressed in *Xenopus* oocytes. The current traces were recorded by applying hyperpolarizing and depolarizing voltage steps (step potentials were from −120 to 10 mV from a holding potential of −110 mV). Ba$_{2+}$ was used as the charge carrier and the concentrations of the bath solution are indicated. B: the current–voltage (I–V) relations of the peak current were plotted as a function of voltage. Changes in the magnitude of the current were observed as the external Ba$_{2+}$ concentration was increased. A shift in the profile of the I–V relation toward positive potentials as the external Ba$_{2+}$ concentration was increased is consistent with increased surface charge screening effects of the divalent cation. C: the current magnitudes of the Ca$_{3.1}$ channel were determined using different concentrations of Ba$_{2+}$, Ca$_{2+}$, and Sr$_{2+}$ (2–65 mM). The solid lines represent fits with a Langmuir isotherm of the form $I = I_{\text{max}}/(1 + K_D/[\text{ion}])$, where $I$ and $I_{\text{max}}$ represent the whole cell current and maximum current, respectively, and [ion] is the divalent ion concentration. The estimated apparent $K_D$ (in mM) values for the Ca$_{3.1}$ were: 4.6 ± 0.6, 4.2 ± 0.4, and 3.1 ± 0.3 (n = 6), for Ba$_{2+}$, Ca$_{2+}$, and Sr$_{2+}$, respectively.
As shown (Fig. 3D), superimposition of the activation (dotted lines) and inactivation (solid lines) curves predicted a window current resulting from overlap of the curves, which ranged from about −60 to −40 mV. Most notably, the peak of the window current of the Sr\(^{2+}\) currents (about −50 mV) was ≥5 mV more negative than the Ba\(^{2+}\) and Ca\(^{2+}\) currents.

Tail currents were elicited with standard pulse protocols to determine the time constants (\(\tau\) values) of deactivation. Then the deactivation \(\tau\) values, the activation \(\tau\) values of Ba\(^{2+}\) current traces, and the relation between step voltages were examined (Fig. 4). One \(\tau\) provided the best fit to the tail currents. The early phase of the tail currents (−0.3 ms) was ignored during curve fitting to remove possible contamination by the capacitive transients. Similar protocols and analyses were used to generate the voltage dependence of activation time constants for Ca\(^{2+}\) and Sr\(^{2+}\) currents (Fig. 4). In another series of experiments, the time dependence of recovery from and development of inactivation of Ba\(^{2+}\) currents at different voltages were determined by use of double-pulse protocols (Fig. 5). Whereas the kinetics of recovery from inactivation were best described with at least two time constants, one time constant was sufficient to fit the profile of the development of inactivation of Ba\(^{2+}\) currents (Fig. 5, A and B). Similar results were obtained for Sr\(^{2+}\) currents (data not shown).

The permeation properties of Ca\(_{3.3}\) are slightly atypical compared with other VGCCs. For example, in contrast to most VGCCs, the throughput of Ba\(^{2+}\) is less than that of Ca\(^{2+}\), as shown in Fig. 2. To further examine the mechanism by which divalent cations traverse the Ca\(_{3.1}\) channels in the inner ear, we used mixtures of Ba\(^{2+}/\text{Ca}^{2+}\) and Ba\(^{2+}/\text{Sr}^{2+}\) with a fixed total concentration (2.5 mM). For the Ba\(^{2+}/\text{Ca}^{2+}\) and Ba\(^{2+}/\text{Sr}^{2+}\) mixtures (external pH 7.5), the magnitude of the Ca\(_{3.1}\) current increased monotonically as the mole fraction of the most permant cation increased (Fig. 6, A and B). Changing the voltage at which tail currents were measured did not affect the outcome of the results. Since minor uncorrected variations in the membrane potential of oocytes may produce the appearance of anomalous mole fraction effect (AMFE), we used corrected voltages to assess the data (see METHODS).

As expected for a Ca\(_{3.1}\) current, it was blocked by mibebradil (Fig. 7), with the half-blocking concentration of the drug at around 6 \(\mu\)M (Fig. 7C). Nifedipine (10 \(\mu\)M) blocked about 40% of the inner ear Ca\(_{3.1}\) channel current (Fig. 7, A and B; Lee et al. 2006). The properties and pharmacology of the inner ear Ca\(_{3.1}\) channel currents will require further molecular and
functional characterization. Meanwhile, single-channel recordings were obtained from oocytes injected with Cav3.1 mRNA. To estimate the values of the voltage steps in the cell-attached configurations, oocytes were bathed in solutions containing high K⁺ to clamp the membrane potential at about 0 mV. The patch pipette contained 20 mM Ba²⁺ or Ca²⁺. As shown in Fig. 8A, the resulting single-channel fluctuations displayed inward currents that were invariably transient at test potentials ranging from −110 to −30 mV, from a holding potential of −120 mV. An example of the amplitude histograms used to generate the unitary current amplitude is shown in Fig. 8B. The estimated single-channel conductances (in pS) from the regression line of the I–V relationship (Fig. 8, C and D) were 10.7 ± 0.9 (n = 5) and 13.3 ± 1.1 pS (n = 6), for Ba²⁺ and Ca²⁺, respectively.

Expression of Ca₃.₁ channels in the adult inner ear

We investigated the expression of Ca₃.₁ in the mouse inner ear using immunohistochemistry and immunoelectron microscopy approaches. Positive, moderate-intensity staining of Ca₃.₁ was observed in the neuroepithelial tissues of both the cochlea and vestibule (Figs. 9A and 10A) in the adult mouse. In the cristae ampullaris of the semicircular canals and the macula of the utricle and saccule, the immunoreactivity was most intense in the apical portions of the hair cells and their supporting cells (Fig. 9, B and C). Both type I and type II vestibular hair cells showed light reactivity in the cytosol with increasing reactivity at the apical portion underlying the cuticular plate. However, as best shown in Fig. 9B, the...
nerve chalice surrounding the lower portion of the type I vestibular hair cell lacks reactivity, although the supporting cells appeared slightly reactive for Ca\textsubscript{3.1}. At the light microscope level, the supporting cell can be differentiated from type II hair cells by the presence of the dark reticular laminar material just beneath the apical surface of the supporting cell. The transitional zone adjacent to the vestibular hair cells and the dark cell region were positive for Ca\textsubscript{3.1}.

In the adult cochlea, the neurosensory tissue expresses Ca\textsubscript{3.1}. In the organ of Corti (Fig. 10), the immunoreactivity for Ca\textsubscript{3.1} was detected in the OHCs and in the region of Deiter’s cup (Fig. 10A). Dark, punctate labeling was often observed around the nucleus of the OHCs. In contrast, diffuse weak reactivity was noted in the region of the IHCs. The nonsensory regions of the cochlea showed equal or greater reactivity for Ca\textsubscript{3.1} than the cochlear hair cells. Moderate to strong levels of immunoreactivity for Ca\textsubscript{3.1} were found in different nonsensory regions of the adult mouse cochlea (Fig. 11A). The lower type I fibrocyte showed moderate reactivity (Fig. 11, A and B, arrow), whereas the type V fibrocytes (Fig. 11A) of the spiral ligament displayed less intense reactivity. The reactivity appeared to be limited to the deeper versus superficial type V fibrocytes. In many of the cochlear sections, and as shown in the lower-power overview (Fig. 11A), the root cells (asterisk) were immunopositive. In the apical turn of the cochlea, which lacks root cells, the external sulcus epithelia (not shown) were moderately reactive for Ca\textsubscript{3.1}. The remaining sites of reactivity in the cochlear lateral wall were the basal cells of the stria vascularis (Fig. 11C) that,

**FIG. 6.** Permeation properties of Ca\textsubscript{3.1} in the inner ear. Anomalous mole fraction effect (AMFE) is absent in Ca\textsubscript{3.1}. Peak tail currents were measured in mixtures of Ca\textsuperscript{2+} and Ba\textsuperscript{2+}, in which the concentration of divalent ions was kept constant at 2.5 mM. RNA-injected oocytes were held at −110 mV, stepped to −30 mV, and the tail currents were measured at −40 mV (see insets A and B). A: no AMFE was observed in 2.5 mM Ca\textsuperscript{2+}/Ba\textsuperscript{2+} (n = 7). Ca\textsubscript{3.1} currents increased monotonically as a function of the Ba\textsuperscript{2+} mole fraction. The absence of an AMFE was observed at all tested voltages (−70 to −20 mV). B: using 2.5 mM Ba\textsuperscript{2+}/Sr\textsuperscript{2+}, and applying similar protocols, evaluation of the tail currents vs. the fraction produces a monotonic increase in tail currents as a function of the Ba\textsuperscript{2+} mole fraction (n = 7).

**FIG. 7.** Pharmacology of Ca\textsubscript{3.1} current. A: current traces were recorded from Ca\textsubscript{3.1} RNA-injected oocytes. They were held at a holding potential of −110 mV and stepped to different depolarizing voltages. Ba\textsuperscript{2+} was the charge carrier in these experiments. For the example shown, after application of 15 \textmu M mibefradil (mibe), the current was completely blocked. Additionally, 10 \textmu M nimodipine reduced the current magnitude by about 2-fold. B: the I–V relationship of currents in magnitude, showing the effects of 15 \textmu M mibefradil and 10 \textmu M nimodipine on control currents and current recovery after washout. C: mibefradil block of the transient Ba\textsuperscript{2+} current was dose dependent. The half-blocking concentration of mibefradil was estimated to be 5.8 ± 0.6 \textmu M (n = 7).
like the fibrocytes, are of mesenchymal origin. The marginal cells and the intermediate cells did not express Cav3.1 during development or in adulthood. On the medial side of the organ of Corti, the spiral limbus was reactive for Cav3.1 (Fig. 11A, arrows). The staining was more pronounced in the upper region of the spiral limbus (white asterisk), but did not include the interdental cells, an epithelial cell type.

Expression of Cav3.1 channels in the inner ear during development

Immunoreactivity for Cav3.1 was first detected in the embryonic inner ear at embryonic day 18 (E18; Fig. 9, D and E). Virtually all of the epithelial cells lining the membranous labyrinth of the vestibular portion of the inner ear express Cav3.1. Interestingly, the intensity of the reactivity was greater in the nonsensory regions than that in the immature neurosensory epithelium. A light-moderate expression of Cav3.1 was noted in the epithelial cells determined to become the transitional cells, as well as in dark cells and the membrane separating endolymph and perilymph. Whereas in the immature hair cells and supporting cells of the cristae ampullaris (Fig. 9D) and macule of the saccule (Fig. 9E), the reactivity was barely above background. However, the immature hair cells of the cochlea (Fig. 10B) show the same level of reactivity as do the immature support cells of the organ of Corti and the outer sulcus epithelium. The cuboidal epithelial cells, which demarcate the future stria vascularis, lack reactivity. As the hair cells of both the vestibule and cochlea mature, a light, sometimes punctate reactivity for Cav3.1 can be detected throughout the cytosol from E18 to P6 (Figs. 9, F and G and 10C). At P6, the OHCs of the cochlea display Cav3.1 labeling primarily along the plasmalemma (Fig. 10D), whereas the other hair-cell types of the inner ear continue to show cytosolic staining (Figs. 9, H and I and 10D).

The differences in the degree and location of Cav3.1 immunoreactivity between the vestibule and cochlea as well as between sensory and nonsensory tissues continue with increasing maturation of the inner ear. At P8, a moderate reactivity occurs in the perinuclear region of the type I and type II vestibular hair cells, as well as at the apically located cuticular plate. The supporting cells between the hair cells continue to display light-moderate reactivity throughout the cytosol with increasingly greater reactivity at their apical reticular membrane (Fig. 9, J and K). However, reactivity decreases in the transitional zone and in the dark cells so that they show negative Cav3.1 reactivity by P8 (not shown). The nerve chalice surrounding the type I hair cell was also negative for staining. With increasing age beyond P8, the intensity of the reactivity of the Cav3.1 at the reticular membrane of the supporting cell and hair cells increases, and until P18 it is equivalent to that seen in the adult mouse (Fig. 9, B and C).

The immature hair cells of the P8 cochlea show a moderate level of reactivity (Fig. 10D, inset) throughout the cytosol of the IHC and in the subnuclear region of the OHCs. At this age reactivity is also noted in the apical “cup” region of the Deiters’s cell under the base of the OHC, as well as in the epithelial cells of the newly formed inner sulcus. In addition, the fibrocytes of the spiral limbus and ligament begin to show immunoreactivity. Expression of Cav3.1 continues to be noted in the nonsensory cells (Hensen’s and Claudius’) of the organ of Corti as well as the outer sulcus cells. However, as the cochlear tissues continue to mature, reactivity in these cells declines and is virtually at the level of background by P21. During this same time period (P8–P21), the level of immunoreactivity in the fibrocytes of the spiral ligament and limbus increases to exceed that of the cochlear hair cells. At P2, the pattern of Cav3.1 immunoreactivity in the cochlea assumes that shown in Fig. 11A for the mature, adult cochlea.
Ca\textsuperscript{2+} influx through low-voltage-activated T-type Ca\textsuperscript{2+} channels serves multiple roles: they are responsible for spontaneous activity in neurons (Huguenard 1996; Llinás and Yarom 1981) and pacemaker cells (Hagiwara et al. 1988), as well as Ca\textsuperscript{2+}-dependent signaling, including cell proliferation (Rodman et al. 2005a,b), differentiation (Bertolesi et al. 2003; Strobeck et al. 1999), and apoptosis (Wang et al. 1999). We have identified and isolated a novel Cav3.1 channel isoform from the mouse inner ear: a splice variant that excludes exon14, exon25, exon34, and exon35, but includes exon26 and exon38 (Fig. 1). Previous studies have shown that Cav3.1 channels are subject to extensive alternative RNA splicing. So far, 30 distinct splice variants have been identified in the fetal and adult human brain (Emerick et al. 2006; Mittman et al. 1999; Monteil et al. 2000; Murbartian et al. 2004; Yunker and McEnery 2003), suggesting a fundamental mechanism to confer the diversity of T-type Ca\textsuperscript{2+} currents. However, the import of alternative splicing is less understood in mice; only two full-length splice variants of Cav3.1 channels have been reported to date (Mittman et al. 1999; Murbartian et al. 2004; Yunker and McEnery 2003). The inner ear Cav3.1 has distinct utilization of exons from those of the Cav3.1 channels found in mouse brain (BC057399 and AJ012569).

One of the Cav3.1 channels (BC057399) excludes exon8 and encodes a nonfunctional channel lacking half of the pore-forming helix in the first functional domain (IS6). Compared with the other mouse Cav3.1 channels (AJ012569), the inner ear Cav3.1 has short I–II and II–III linkers because of the exclusion of exon14 and exon25A (Fig. 1). Exon14 introduces an additional 23 aas into the II–III loop of the channel. Splice variants with this insert display faster inactivation kinetics

**FIG. 9.** Cav3.1 expression in the vestibular system. **A:** in the adult, Cav3.1 is expressed in all vestibular organs (arrow) and, to a lesser degree, in the underlying connective tissue (asterisk). **B** and **C:** higher-power views of the saccule (**B**) and ampulla (**C**) show staining in the apical portion of support cells and hair cells (**B**, between arrowheads). **D** and **E:** Cav3.1 is first noted at embryonic day 18 (E18) in all epithelial (asterisk) and neuroepithelial cells (arrows) in the membranous labyrinth. **F** and **G:** at postnatal day 0 (P0), the cytosol of macular and support cells (asterisk) showed light reactivity. **H** and **I:** by P6, a moderate immunoreactivity can be seen in the cytosol of hair cells (arrowheads) and support cells. The nerve chalice at the type I hair cells is negative (asterisk). **J** and **K:** by P8, moderately dark reactivity was seen in apices of hair cells and support cells (arrows). The type I perinuclear region shows light reactivity (arrowhead), whereas the afferent nerve chalice is negative.

**DISCUSSION**

Ca\textsuperscript{2+} influx through low-voltage-activated T-type Ca\textsuperscript{2+} channels serves multiple roles: they are responsible for spontaneous activity in neurons (Huguenard 1996; Llinás and Yarom 1981) and pacemaker cells (Hagiwara et al. 1988), as well as Ca\textsuperscript{2+}-dependent signaling, including cell proliferation (Rodman et al. 2005a,b), differentiation (Bertolesi et al. 2003; Strobeck et al. 1999), and apoptosis (Wang et al. 1999). We have identified and isolated a novel Cav3.1 channel isoform from the mouse inner ear: a splice variant that excludes exon14, exon25, exon34, and exon35, but includes exon26 and exon38 (Fig. 1). Previous studies have shown that Cav3.1 channels are subject to extensive alternative RNA splicing. So far, >30 distinct splice variants have been identified in the fetal and adult human brain (Emerick et al. 2006; Mittman et al. 1999; Monteil et al. 2000; Murbartian et al. 2004; Yunker and McEnery 2003), suggesting a fundamental mechanism to confer the diversity of T-type Ca\textsuperscript{2+} currents. However, the import of alternative splicing is less understood in mice; only two full-length splice variants of Cav3.1 channels have been reported to date (Mittman et al. 1999; Murbartian et al. 2004; Yunker and McEnery 2003). The inner ear Cav3.1 has distinct utilization of exons from those of the Cav3.1 channels found in mouse brain (BC057399 and AJ012569).

One of the Cav3.1 channels (BC057399) excludes exon8 and encodes a nonfunctional channel lacking half of the pore-forming helix in the first functional domain (IS6). Compared with the other mouse Cav3.1 channels (AJ012569), the inner ear Cav3.1 has short I–II and II–III linkers because of the exclusion of exon14 and exon25A (Fig. 1). Exon14 introduces an additional 23 aas into the II–III loop of the channel. Splice variants with this insert display faster inactivation kinetics
compared with channels without. Moreover, Ca\textsubscript{3.1} channels including exon25A, which encodes an extra stretch of 7 aas in the intracellular III–IV loop, activate at more negative membrane potentials and show a faster activation rate compared with that of other Ca\textsubscript{3.1} channels (Chemin et al. 2001). As predicted based on their structure, the inner ear and other mouse Ca\textsubscript{3.1} channels differ in their molecular and electrophysiologic properties.

The primary structure of the inner ear Ca\textsubscript{3.1} is distinct from that of other Ca\textsubscript{3.1} channels at several important functional domains. The lack of exon14 excludes a consensu phosphorilation site for protein kinase C in the intracellular II–III loop, which may serve as an important regulatory site (Chemin et al. 2001). Similarly, the inner ear Ca\textsubscript{3.1} channel also lacks a casein kinase II phosphorylation site in the intracellular III–IV loop, encoded by exon34. Again, these differences reinforce the potential for differential regulatory mechanisms between the inner ear and other Ca\textsubscript{3.1} channels. Although the biological significace of these splice variants is not completely understood, the differences in their physiological functions, together with their tissue-specific distribution, may contribute to differential regulation and modulation of biological processes, resulting in tissue-specific intracellular Ca\textsuperscript{2+} handling.

The kinetic phenotype of the inner ear Ca\textsubscript{3.1} channels in heterologous expression system, with respect to the fast inactivation, was distinct from typical Ca\textsuperscript{2+} currents recorded from developing hair cells (Levic et al. 2007). Moreover, the results were not surprising since recapitulation of the native cell current properties invariably requires auxiliary subunits (Dolphin et al. 1999; Lacerda et al. 1994; Leuranguer et al. 1998). The sensitivity of the channel to nimodipine may explain why the current has been missed in previous studies in hair cells (Rodriguez-Contreras and Yamoah 2003).

Ca\textsuperscript{2+} influx from T-type Ca\textsuperscript{2+} currents gives rise to many scenarios for developing and regenerating hair cells (Levic et al. 2007). Membrane excitability is promoted when the low-threshold features of the current amplify the depolarizing inputs. Inactivation of this current may generate burst firing patterns from tonic inputs. As a result, Ca\textsuperscript{2+} entry driven by a large tail current follows to facilitate a robust change of intracellular Ca\textsuperscript{2+} during activity. Additionally, steady Ca\textsuperscript{2+} influx will occur due to a window current caused by the overlap of the steady-state inactivation and activation relations at a potential range near the resting potential of hair cells. This steady inward flow of Ca\textsuperscript{2+} will result in a further boost of intracellular Ca\textsuperscript{2+} (Barish and Mansdorf 1991). Last, Ca\textsuperscript{2+} current inactivation may be self-limiting, creating a feedback control to prevent Ca\textsuperscript{2+} overload. Functional studies have
shown that T-type currents can mediate phasic changes in intracellular Ca\textsuperscript{2+}, which may prove to be an important requirement for the synthesis and release of neurotrophins during hair-cell development and regeneration (Eatock and Hurley 2003). Patterned electrical activity and the ensuing changes in Ca\textsuperscript{2+} transients, opposed to steady firing activity, preferentially stimulate the transcription and secretion of neurotrophins (Balkowiec and Katz 2002). Moreover, electrical activity tightly regulates the expression of neurotrophin receptors, giving insight into a mechanism by which neurotrophins may selectively affect electrically active neurons. In particular, there is a solid link between the release of brain-derived neurotrophic factor and neurotrophin 3 and the specificity of hair-cell innervation in the cochlea of the inner ear. A base-to-apex preference gradient is created when neurotrophins define the innervation pattern of type II afferents on OHCs (Farinas et al. 2001).

Tissue-specific localization of T-type Ca\textsuperscript{2+} channels has prompted the suggestion that the channel subtypes play unique roles that cannot be compensated by other channels (Talley et al. 1999). Because mibebradil inhibits cell proliferation in vascular tissues and there appears to be robust expression of T-type current as cells transition from G0 to G1 and S-phase, the possibility that T-type channels could be involved in regulating cell proliferation has been raised (Bertolesi et al. 2003). Localization of the Ca\textsubscript{3.1} immunoreactivity to nonsensory cells of the cochlea, vestibule, and fibrocytes of the spiral ligament and limbus is striking. The correlation between the expression of Ca\textsubscript{3.1} and the propensity of nonsensory cells to proliferate (Holley 2005) is also remarkable and it is conceivable that the Ca\textsubscript{3.1} channels in cells in the inner ear may be an index for cell division. Indeed, a recent report has demonstrated the presence of a T-type current in developing hair cells (Levic et al. 2007), which is consistent with the hypothesis that Ca\textsubscript{3.1} may be important for cellular differentiation. Also important, the robust expression of Ca\textsubscript{3.1} and reappearance in regenerating hair cells is in further agreement with the concept that expression of T-type currents begets cellular development. T-type currents are frequently observed in the early development of cells and their density, amplitude, and properties change over time as seen in embryonic dorsal root ganglia, retinal Müller cells, hippocampal neurons, and thalamocortical cells (Bringmann et al. 2000; Desmadryl et al. 1998; Pirchio et al. 1990; Yaari et al. 1987). Moreover, in some cell types, T-type current density decreases with age until a mature stage is reached. At that time the current density is reduced substantially, giving way to the expression of high-voltage–activated (HVA) Ca\textsuperscript{2+} currents (Chameau et al. 1999). Indeed, it has been reported that T-type currents may regulate the expression of HVA Ca\textsuperscript{2+} currents (Chemin et al. 2002b), raising the

FIG. 11. Ca\textsubscript{3.1} in nonsensory tissues. A: the basal turn of the cochlea revealed moderate immunopositivity for Ca\textsubscript{3.1} in fibrocytes and root cells of the spiral ligament (Sp Lig) (asterisks). Unlike the ID cells, the upper fibrocytes of the spiral limbus (white asterisk) showed strong reactivity with less intense staining noted in the lower spiral limbal fibrocytes (arrows). B: higher magnification of the spiral ligament revealed moderate positivity in lower type I fibrocytes (arrow) with less reactivity in the upper type I fibrocytes underlying the stria vascularis (StV). C: basal cells (BCs) of the stria vascularis (arrows) showed moderate reactivity. D: as positive control, we show strong reactivity for Ca\textsubscript{3.1} in the Purkinje (P) layer of the cerebellum (arrows; Nahm et al. 2005). E: substitution of nonimmune serum and/or antigenic peptide for the Ca\textsubscript{3.1} antibody failed to show any reactivity in the crista ampullaris (asterisk). OHC, outer hair cell; IHC, inner hair cell; IC, intermediate cell; MC, marginal cell; DC, dark cell.
possibility of mechanisms for coordination of T-type and HVA Ca\(^{2+}\) channel expression in hair cells.

REFERENCES


