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Electrical Properties and Functional Expression of Ionic Channels in Cochlear Inner Hair Cells of Mice Lacking the α10 Nicotinic Cholinergic Receptor Subunit

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ABSTRACT

Cochlear inner hair cells (IHCs) release neurotransmitter onto afferent auditory nerve fibers in response to sound stimulation. During early development, synaptic transmission is triggered by spontaneous Ca^{2+} spikes which are modulated by an efferent cholinergic innervation to IHCs. This synapse is inhibitory and mediated by the α9α10 nicotinic cholinergic receptor (nAChR). After the onset of hearing, large-conductance Ca^{2+}-activated K^+ channels are acquired and both the spiking activity and the efferent innervation disappear from IHCs. In this work, we studied the developmental changes in the membrane properties of cochlear IHCs from α10 nAChR gene (*Chrna10*) "knockout" mice. Electrophysiological properties of IHCs were studied by whole-cell recordings in acutely excised apical turns of the organ of Corti from developing mice. Neither the spiking activity nor the developmental functional expression of voltage-gated and/or calcium-sensitive K^+ channels is altered in the absence of the α10 nAChR subunit. The present results show that the α10 nAChR subunit is not essential for the correct establishment of the intrinsic electrical properties of IHCs during development.

Keywords: calcium spikes, voltage-gated K^+ channels, calcium-sensitive K^+ channels, cholinergic, development, auditory

INTRODUCTION

Inner hair cells (IHCs) are mechanosensory cells that encode incoming acoustic stimuli in the mammalian cochlea. In rodents, hearing function begins in the second postnatal (P) week. Embryonic and neonatal IHC have functional transduction channels (Kros et al. 1992; Eatock 2003; Housley et al. 2006), voltage-gated K^+ channels (Kros et al. 1998; Marcotti et al. 2003a), calcium-activated SK2 potassium channels (Glowatzki and Fuchs 2000; Katz et al. 2004; Marcotti et al. 2004; Goutman et al. 2005), and also voltage-gated Na^+ (Kros et al. 1992; Marcotti et al. 2003b) and Ca^{2+} channels (Platzer et al. 2000; Marcotti et al. 2003b; Raffaelli et al. 2004; Johnson and Marcotti 2008). Together, these ion channels underlie, shape, and sustain Ca^{2+} spiking activity (Kros et al. 1998; Marcotti et al. 2003b, 2004; Housley et al. 2006). This spiking activity drives synaptic transmission before the onset of hearing (Beutner and Moser 2001; Johnson et al. 2007) and may trigger rhythmic or bursting activity of neurons at higher levels of the auditory pathway (Gummer and Mark 1994; Kotak and Sanes

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1995; Eatock 2003). After the onset of hearing (postnatal days 12–13 in mice), spiking completely disappears, mainly due to the expression of a fast K^+ current carried by calcium-activated BK channels (Kros and Crawford 1990; Marcotti et al. 2003a).

Before the onset of hearing, olivocochlear fibers transiently innervate IHCs (Simmons et al. 1996; Pujol et al. 1998). The function of this innervation is still unresolved, but it may play a role in the rhythmic firing of immature auditory afferents (Puel 1995; Walsh et al. 1998; Glowatzki and Fuchs 2000; Goutman et al. 2005). Clearly, they can suppress the excitability of IHCs and presumably prevent transmitter release from ribbon synapses known to be functional at this stage (Glowatzki and Fuchs 2000; Glowatzki and Fuchs 2002; Goutman et al. 2005).

This synapse is inhibitory and is mediated by the α9α10 nicotinic acetylcholine (ACh) receptor (nAChR; Elgoyhen et al. 1994, 2001; Vetter et al. 1999, 2007; Glowatzki and Fuchs 2000; Weisstaub et al. 2002; Gomez-Casati et al. 2005). Activation of the α9α10 nAChR leads to an increase in intracellular Ca^{2+} and the subsequent opening of SK2 channels (Fuchs and Murrow 1992; Blanchet et al. 1996; Dulon et al. 1998; Oliver et al. 2000; Katz et al. 2004; Marcotti et al. 2004; Nemzou et al. 2006) that hyperpolarize the cells. IHCs express messenger RNA (mRNA) for both α9 and α10 nAChRs subunits beginning in late embryogenesis. The expression of *Chrna10*, but not that of *Chrna9* (Elgoyhen et al. 1994, 2001; Simmons 2002), as well as that of SK2 channels (Katz et al. 2004), drop to undetectable levels in IHCs after the second postnatal week (~P13–14). At the same time, both cholinergic sensitivity (Katz et al. 2004) and efferent innervation disappear from these cells (Liberman et al. 1990; Simmons 2002).

The genetic ablation of the SK2 channel has a dramatic effect on the efferent innervation and in the cholinergic sensitivity of cochlear hair cells (Kong et al. 2008; Murthy et al. 2009). In addition, IHCs from SK2 knockout mice are unable to sustain calcium spiking activity (Marcotti et al. 2004; Johnson et al. 2007). These mutants, however, do not present modifications in the developmental expression of other potassium currents (Johnson et al. 2007; Kong et al. 2008). Considering the dramatic effects that SK2 knockout mice have on the olivocochlear system and on previous work showing a tight dynamic regulation of *Chrna10* gene expression in IHCs during synaptogenesis (Elgoyhen et al. 2001; Simmons 2002; Katz et al. 2004), we hypothesized that hair cell physiology might be affected by the lack of expression of the *Chrna10* gene. We found, however, that neither the spiking activity nor the developmental functional expression of voltage-gated and calcium sensitive K^+ channels are altered by the absence of the α10 nAChR subunit.

METHODS

Animal procedures and isolation of the organ of Corti

B6Cast mice (N8-N10; *Chrna10*$^{+/+}$, *Chrna10*$^{+/-}$, and *Chrna10*$^{-/-}$), at seven to 17 postnatal days (P7–17; day of birth was considered P0), were anesthetized using pentobarbital and decapitated. All experimental protocols were carried out in accordance with the National Institute of Health guide for the care and use of Laboratory animals (NIH Publications no. 80-23) revised 1978. The organ of Corti was exposed and the apical turns were removed for recording as previously described (Glowatzki and Fuchs 2000; Katz et al. 2004; Gomez-Casati et al. 2005) and used within 3 h. Cochlear preparations were mounted under a Leica DMLFS microscope (Leica, D-35578 Wetzlar, Germany) and viewed with differential interference contrast using a 40x water immersion objective and a camera with contrast enhancement (Hamamatsu C2741, Hamamatsu City, Japan).

Electrophysiological recordings

Methods to record from IHCs were essentially as described (Glowatzki and Fuchs 2000; Katz et al. 2004; Gomez-Casati et al. 2005). Briefly, IHCs were first identified visually and then by their whole-cell capacitance (7 to 12 pF) and their characteristic voltage-dependent Na^+ and K^+ currents, including at older ages a fast-activating K^+ conductance (Kros et al. 1998; Marcotti et al. 2003a). Some cells were removed to access IHCs, but mostly, the pipette electrodes were advanced through the tissue under positive pipette pressure.

Currents in IHCs were recorded in the whole-cell patch-clamp configuration using an Axopatch 200B amplifier (Molecular Devices, Sunny Vale, CA 94089, USA), low-pass-filtered at 2–10 kHz, and digitized at 5–20 kHz with a Digidata 1200 board (Molecular Devices). Recordings were made at room temperature (22–25°C). Patch pipettes were pulled from Corning 7056 glass capillaries (Warner Inst. Inc., Hamden, CT 06514, USA), and electrode resistances in the extracellular solution were 5–7 MΩ . In order to reduce the electrode capacitance, the shank of the electrode was coated with sylgard. Working solutions were applied by a gravity-fed multi-channel glass pipette (~150-μM tip diameter) positioned about 300 μm from the recorded IHC. In voltage clamp recordings, series resistance ($R_s \leq 10$–12 MΩ) was compensated on line 0–60% (most cells only up to 40%). Currents

were not corrected for the residual R_s. Therefore, time constants of activation could be somewhat underestimated. In addition, due to IR drop caused by the flow of currents across the uncompensated R_s, we would also be making an error in the voltage at which we are measuring the currents and therefore underestimating them, particularly in the case of large currents. Linear leak currents were subtracted off-line: leak conductance was calculated between −84 and −94 mV. The indicated holding potentials were corrected off-line for the liquid junction potential (−4 mV) using the PClamp9 specific software.

Solutions

The extracellular solution was as follows (in mM): 155 NaCl, 5.8 KCl, 1.3 $CaCl_2$, 0.9 $MgCl_2$, 0.7 NaH_2PO_4, 5.6 D-glucose, and 10 HEPES buffer; pH was adjusted to 7.4 with NaOH (the osmolarity was about 300–310 mOsm/kg). The pipette solutions contained (in mM): 150 KCl, 3.5 $MgCl_2$, 0.1 $CaCl_2$, 5 or 1 ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 5 Hepes buffer, 2.5 Na_2ATP; pH was adjusted to 7.2 with KOH. The osmolarity of intracellular solutions ranged between 270 and 290 mOsm/kg. SK2-sensitive currents in IHCs were recorded using an intracellular solution containing 1 mM EGTA. Action potentials were recorded immediately after rupturing into the cell.

DATA ANALYSIS

Statistical analyses were carried by analysis of variance (ANOVA) followed by a Tukey's test for multiple comparisons. A value of $p<0.05$ was selected as the criterion for statistical significance. Mean values are quoted as means±SEM.

MATERIALS

ACh chloride, apamin, Na_2ATP, and all other reagents were from Sigma Chemical Co. (St. Louis, MO, USA). EGTA and Na_2ATP were dissolved at the moment of preparing the intracellular solutions. The other drugs were dissolved in distilled water as 1–10 or 100 mM stocks and stored in aliquots at −20°C.

RESULTS

Spiking activity in IHCs of *Chrna10*$^{-/-}$ mice

Early postnatal IHCs can generate slowly repetitive calcium action potentials spontaneously or during injection of small depolarizing currents (Kros et al. 1998; Glowatzki and Fuchs 2000; Marcotti et al. 2003b). This firing activity is blocked by ACh acting on α9α10 nAChR (Glowatzki and Fuchs 2000; Goutman et al. 2005). To evaluate whether the lack of expression of a functional α9α10 nAChR in IHCs could alter spiking, we examined if IHCs from *Chrna10*$^{-/-}$ mice were able to generate calcium action potentials before the onset of hearing. As previously reported (Marcotti et al. 2004), at P7–9, spontaneous firing was found very infrequently (only in one cell out of six in *Chrna10*$^{+/+}$ and never in *Chrna10*$^{+/-}$ and *Chrna10*$^{-/-}$ mice (eight and 14 cells tested, respectively). Therefore, in order to evaluate whether the lack of the α10 subunit affected spiking, we examined action potential features such as threshold, amplitude, duration, and frequency by injecting small depolarizing currents to P7–9 IHCs from *Chrna10*$^{+/+}$ and *Chrna10*$^{-/-}$ mice in the whole-cell current clamp configuration. Figure 1A illustrates slowly repetitive calcium action potentials elicited by a constant injected current of 50 and 70 pA in *Chrna10*$^{+/+}$ and *Chrna10*$^{-/-}$ mice (n=5 and 10 IHCs, respectively) before the onset of hearing. There were no consistent differences in the frequency of action potentials between genotypes (Table 1). Moreover, the amplitude, half-width, and threshold of calcium spikes did not differ between IHCs from *Chrna10*$^{+/+}$ and *Chrna10*$^{-/-}$ P7–9 mice (Table 1). As can be observed in Figure 1B, superimposed averaged traces of calcium spikes evoked by 70 pA in IHCs from *Chrna10*$^{+/+}$ and *Chrna10*$^{-/-}$ mice had an identical temporal course (Fig. 1B). This suggested that the Ca^{2+}, Na^+, and K^+ currents involved in generating and shaping these action potentials (Marcotti et al. 2003b, 2004; Housley et al. 2006) might not be affected by the genetic ablation of the α10 subunit. In addition, no differences between genotypes were found in cell capacitance and resting membrane potential either before or after the onset of hearing (Table 2).

It has been shown that efferent activity can delay or prevent calcium action potentials in neonatal rat IHCs (Glowatzki and Fuchs 2000; Goutman et al. 2005). We thus examined the effect of exogenously applied ACh on the spiking activity of these cells. Consistent with the notion that in IHCs no functional nAChR channels are formed in the absence of the α10 subunit and due to which cholinergic sensitivity is lost (Vetter et al. 2007), 1 mM ACh was not able to abolish the generation of calcium action potentials in IHCs from *Chrna10*$^{-/-}$ mice (Fig. 1D, n=5 IHCs). Contrastingly, action potential activity was blocked in all IHCs tested from *Chrna10*$^{+/+}$ mice (Fig. 1D, n=5 IHCs). In addition, calcium action potentials were also blocked in

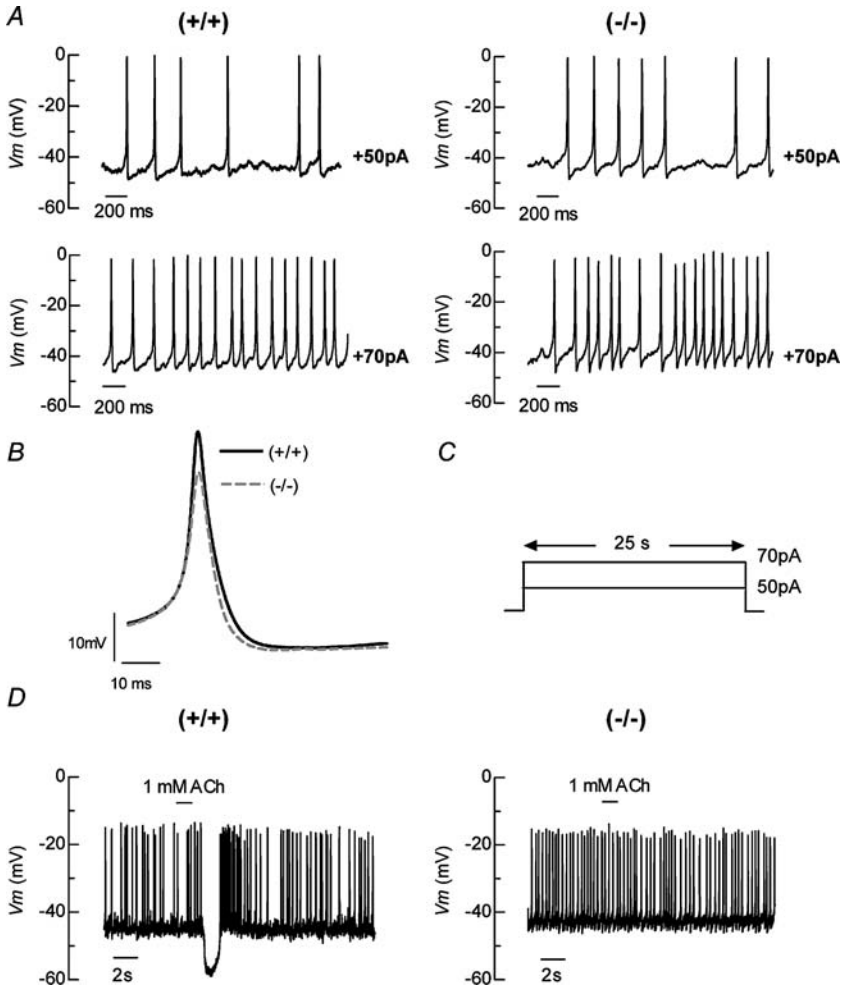


FIG. 1. IHC voltage responses before the onset of hearing. **A** Representative voltage responses under current clamp from apical coil IHCs before the onset of hearing in *Chrna10*$^{+/+}$ and *Chrna10*$^{-/-}$ mice. To elicit frequent firing of calcium action potentials, we constantly injected a depolarizing current (see protocol in **C**) into *Chrna10*$^{+/+}$ and *Chrna10*$^{-/-}$ IHCs (n=5 and 10 cells, respectively). **B** Superimposed average of evoked (+70 pA) calcium action potentials from wild-type (*black*; 524 events, five cells) and α10 knockout (*gray*; 877 events, ten cells) mice showing that there are no differences in the temporal course of the calcium action potentials between both genotypes (see also Table 1). **D** Application of 1 mM ACh hyperpolarized the membrane potential from about −47 to −60 mV and abolished the generation of evoked action potentials in IHCs from both *Chrna10*$^{+/+}$ (n=5 IHCs). In contrast, the application of 1 mM ACh in IHCs from *Chrna10*$^{-/-}$ mice did not affect the resting membrane potential and did not abolish the generation of calcium spikes (n=5 IHCs).

TABLE 1

Action potential parameters

	Chrna10		
	(+/+) (n=5)	*(−/−) (n=10)*	*p value*
Amplitude (mV)	33.6±4.7	27.1±2.0	0.17
Half-width (ms)	6.3±1.0	7.0±0.7	0.59
Frequency (Hz)	4.2±0.4	3.9±0.8	0.76
Threshold (mV)	−40.4±1.4	−36.6±1.1	0.08

Action potentials for this analysis were elicited by a +70 pA current step. Values are the mean±SEM; *n* number of IHC tested. For amplitude, half-width, and frequency, the number of events analyzed were: 524 (*Chrna10*$^{+/+}$) and 877 (*Chrna10*$^{-/-}$) from five and ten IHCs, respectively. Threshold for spiking activity was determined by a 25-ms current ramp from 0 to 90 pA

three out of three IHCs from *Chrna10*$^{+/-}$ mice (n= 3, data not illustrated), thus showing that the presence of one allele is sufficient to drive normal olivocochlear efferent function to these cells.

Slowly activating Ca^{2+}-dependent outward currents in IHCs before the onset of hearing

The inhibitory nature of the olivocochlear synapse in immature IHCs is due to the activation of an SK2 channel after Ca^{2+} influx through the α9α10-containing nAChRs (Glowatzki and Fuchs 2000; Katz et al. 2004; Marcotti et al. 2004; Gomez-Casati et al. 2005). After the onset of hearing, both

TABLE 2

IHC membrane properties

	Chrna10									
	(+/+)			(+/−)			(−/−)		ANOVA (*p* value)	
Age	C_m (pF)	V_m (mV)		C_m (pF)	V_m (mV)		C_m (pF)	V_m (mV)	C_m (pF)	V_m (mV)
P7–9	7.5±0.4 (*n*=9)	−51.2± 3.3 (*n*=9)		8.3±0.3 (*n*=30)	−58.4±1.1 (*n*=30)		8.2±0.3 (*n*=23)	−54.4±1.5 (*n*=23)	0.45	0.06
P14–17	8.3±0.3 (*n*=3)	−64.0±7.0 (*n*=3)		8.6±0.4 (*n*=13)	−66.3±2.4 (*n*=13)		8.0±0.8 (*n*=4)	−66.0±3.4 (*n*=4)	0.74	0.92

Cell capacitance was measured from current transients in response to a pulse of 10 mV from a V_{hold} of −90 mV. The resting membrane potential was measured as zero-current potentials under voltage clamp. The statistics shown in the table correspond to an ANOVA between data obtained in IHCs from *Chrna10*$^{+/+}$, *Chrna10*$^{+/-}$, *Chrna10*$^{-/-}$ mice. C_m and V_m values between P7–9 and P14–17 were not significantly different in any of the three genotypes (p>0.05).

n number of IHCs tested

Chrna10 and *SK2* gene expression is down-regulated, and this is correlated with the disappearance of ACh-evoked responses (Katz et al. 2004). We therefore evaluated the presence of functional SK2 channels in the *Chrna10*$^{-/-}$ mice by promoting Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Marcotti et al. 2004; Kong et al. 2008). When immature IHCs from *Chrna10*$^{+/+}$ mice were depolarized for 4 s from a holding potential of −84 mV (Fig. 2A) using 1 mM EGTA as the intracellular Ca^{2+} buffer and 1.3 mM extracellular Ca^{2+}, a slowly activating outward current was evident in 76% of the wild-type cells tested (Fig. 2B, black traces). Extracellular application of 300 nM apamin, a selective SK2 channel blocker (Kohler et al. 1996), abolished this slowly activating outward K^+ current (gray traces in Fig. 2B and traces shown in Fig. 2C: here records in the presence of apamin were subtracted from control records, leaving only the apamin-sensitive current), suggesting, as previously demonstrated (Marcotti et al. 2004), that this current was due to the activation of SK2 channels as a result of Ca^{2+} influx through voltage-gated calcium channels. When a Ca^{2+}-free extracellular solution (containing 1 mM EGTA) was superfused onto immature IHCs, the slowly activating outward current was selectively and reversibly abolished, providing further evidence for its Ca^{2+} sensitivity (data not shown). The maximum amplitude of the SK2 current measured at steady state (2 s) at 16 mV in IHCs from *Chrna10*$^{-/-}$ mice (335.5±64.1 pA, *n*=6 cells; Fig. 2E) did not differ from that obtained in IHCs from wild-type mice (345.2±73.6 pA, *n*=9 cells; Fig. 2C; p>0.5). This current was no longer evident in IHCs of wild type or *Chrna10*$^{-/-}$ mice after the onset of hearing (Fig. 2F–I).

Total outward K^+ currents expressed in IHCs during development

Depolarizing voltage steps from a holding potential of −84 mV caused slowly developing voltage-dependent outward K^+ currents (a delayed rectifier-type K^+ current named $I_{K,neo}$ for the neonatal cells; Kros et al. 1998; Marcotti et al. 2003a) in all IHCs from the three genotypes before the onset of hearing (Fig. 3B, upper panel). After the onset of hearing, the fast activating Ca^{2+}-dependent K^+ current ($I_{K,f}$), characteristic of mature IHCs, was also observed in IHCs from *Chrna10*$^{+/+}$, *Chrna10*$^{+/-}$, and *Chrna10*$^{-/-}$ mice (Fig. 3B, lower panel). The size of the outward K^+ currents increased between P7–9 and P14–17 in the three genotypes. Current–voltage curves were generated from the amplitudes of total outward currents measured at two time points: at the beginning (Fig. 3C) and at the end (Fig. 3D) of the 170-ms test pulses illustrated

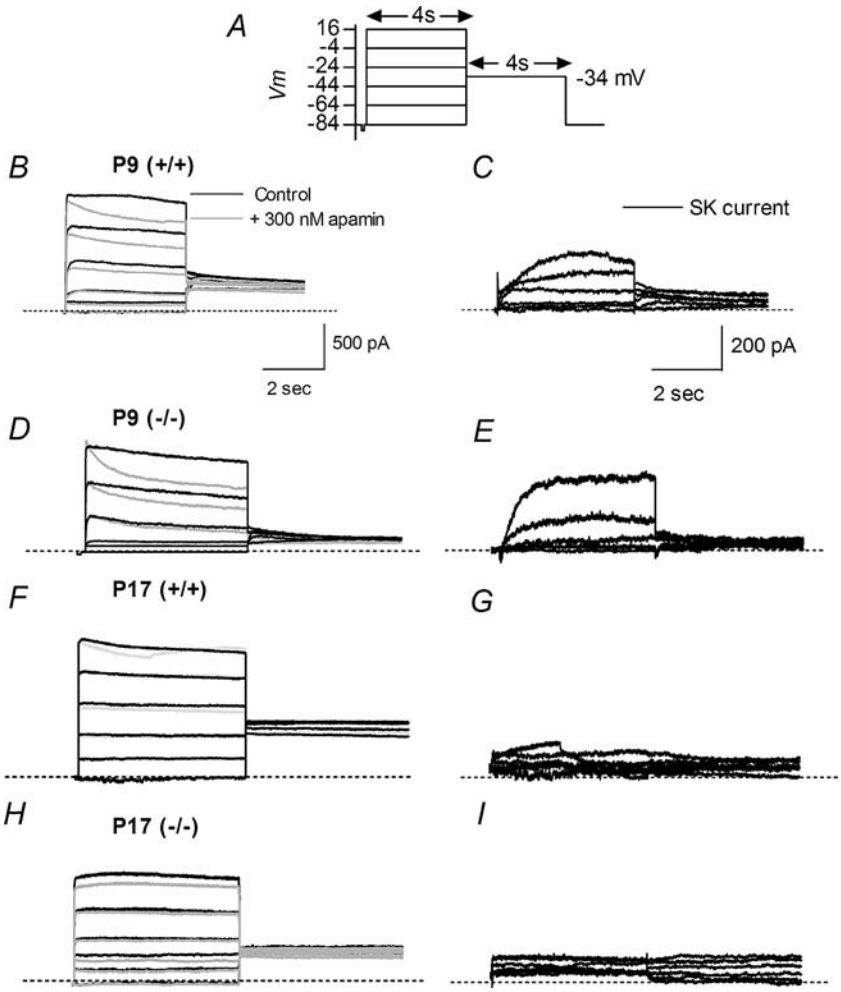


FIG. 2. Development of the slowly activating outward currents in IHCs. **A** Protocol used to measure the slowly activating outward currents in IHCs. Currents were elicited by 4-s depolarizing voltage steps in 20-mV increments from −84 to 16 mV starting from the holding potential of −84 mV. **B** Representative outward currents recorded in IHCs from *Chrna10*$^{+/+}$ mice (P9) in the absence (control) or presence of 300 nM apamin in the extracellular solution. **C** Subtracting the currents recorded in the presence of apamin from the total currents (control) reveals the presence of SK2 currents (n=9 IHCs). **D** Representative outward currents recorded in IHCs from *Chrna10*$^{-/-}$ mice (P9) in the absence (control) or presence of 300 nM apamin in the extracellular solution. **E** Subtracting the currents recorded in the presence of apamin from the total currents (control) reveals the presence of SK2 currents (n=6 IHCs). **F** Representative outward currents recorded from IHCs from P17 wild-type mice in the absence (control) or presence of 300 nM apamin in the extracellular solution. **G** Subtraction of these currents shows that there are no SK2 currents at this age (n=5 IHCs). **H** Representative outward currents recorded from IHCs from *Chrna10*$^{-/-}$ mice at P17 in the absence (control) or presence of 300 nM apamin in the extracellular solution. **I** Subtraction of these currents shows that as in *Chrna10*$^{+/+}$ mice, there are no SK2 currents at this age in the *Chrna10*$^{-/-}$ mice (n=3 IHCs).

in Figure 3A. As was previously described (Kros et al. 1998; Pyott et al. 2004), the fast component corresponding to $I_{K,f}$ was absent at P7–9 and appeared at P14-17 in IHCs from *Chrna10*$^{+/+}$ mice (Fig. 3D and Table 3). Similar results were obtained in IHCs from *Chrna10*$^{+/-}$ and *Chrna10*$^{-/-}$ mice. The activation time constant for P7–9 *Chrna10*$^{-/-}$ mice was somewhat faster (τ=2.4; p<0.05), however, than that of their wild-type and heterozygous littermates (see Table 3). Notwithstanding, this value is much slower than the one that can be explained by the expression of $I_{K,f}$ (Kros et al. 1998).

In the three genotypes, the amplitude of maximal responses (normalized by whole-cell capacitance to account for any systematic changes in cell size) was smaller in the P7–9 group than in the P14–17 group (p<0.05, Fig. 3E). The difference in amplitude between the two age groups was significant for the three genotypes, even though amplitudes at P14–17 could be underestimated by the fact that R_s was only compensated to 40% (see "Methods"). There were no significant differences, however, in the amplitude of maximal responses in IHCs from *Chrna10*$^{+/+}$, *Chrna10*$^{+/-}$, and *Chrna10*$^{-/-}$ in either age group (p>

0.05). Outward currents in P14–17 IHCs from the three genotypes had the fast component previously documented as $I_{K,f}$ (Kros et al. 1998; Pyott et al. 2004; Table 3). The activation rates of currents recorded from P7–9 preparations at a voltage of 46 mV were slower, consistent with the presence of $I_{K,neo}$ (Table 3). Reversal potentials of total outward currents were determined for the three genotypes before and after the onset of hearing and were all close to the K^+ equilibrium potential under our experimental conditions (E_K=−82 mV), confirming that outward currents were mainly carried by K^+ ions (Table 3).

Steady-state activation of total K^+ currents during development

To study whether there were developmental changes in the voltage dependency of activation of total outward K^+ currents in IHCs of *Chrna10*$^{+/+}$, *Chrna10*$^{+/-}$, and *Chrna10*$^{-/-}$ mice, tail currents at a fixed membrane potential were analyzed (Fig. 4). From a holding level of −84 mV, the membrane potential was stepped to a test potential of −44 mV after a series of conditioning depolarizing pulses (170 ms in duration) in 10-mV steps (see protocol in Fig. 3A). The activation curves were obtained by plotting the normalized instantaneous tail currents (measured 0.2–0.4 ms after stepping to the test potential) against the different pre-pulse potentials. Data were fitted by a single first-order Boltzmann equation: $I=I_{max}/1+(\exp\ ((V_{50}-V)/S))$. Tail current amplitude is represented by I, I_{max} is the maximal tail current amplitude, V_{50} is the potential of half-maximal activation, V is the membrane potential of the preceding voltage step, and S describes the voltage sensitivity of activation. Both V_{50} and the slope factor (S) showed no clear trend with age and no differences across genotypes (Fig. 4B, C).

Inward rectifier K^+ current

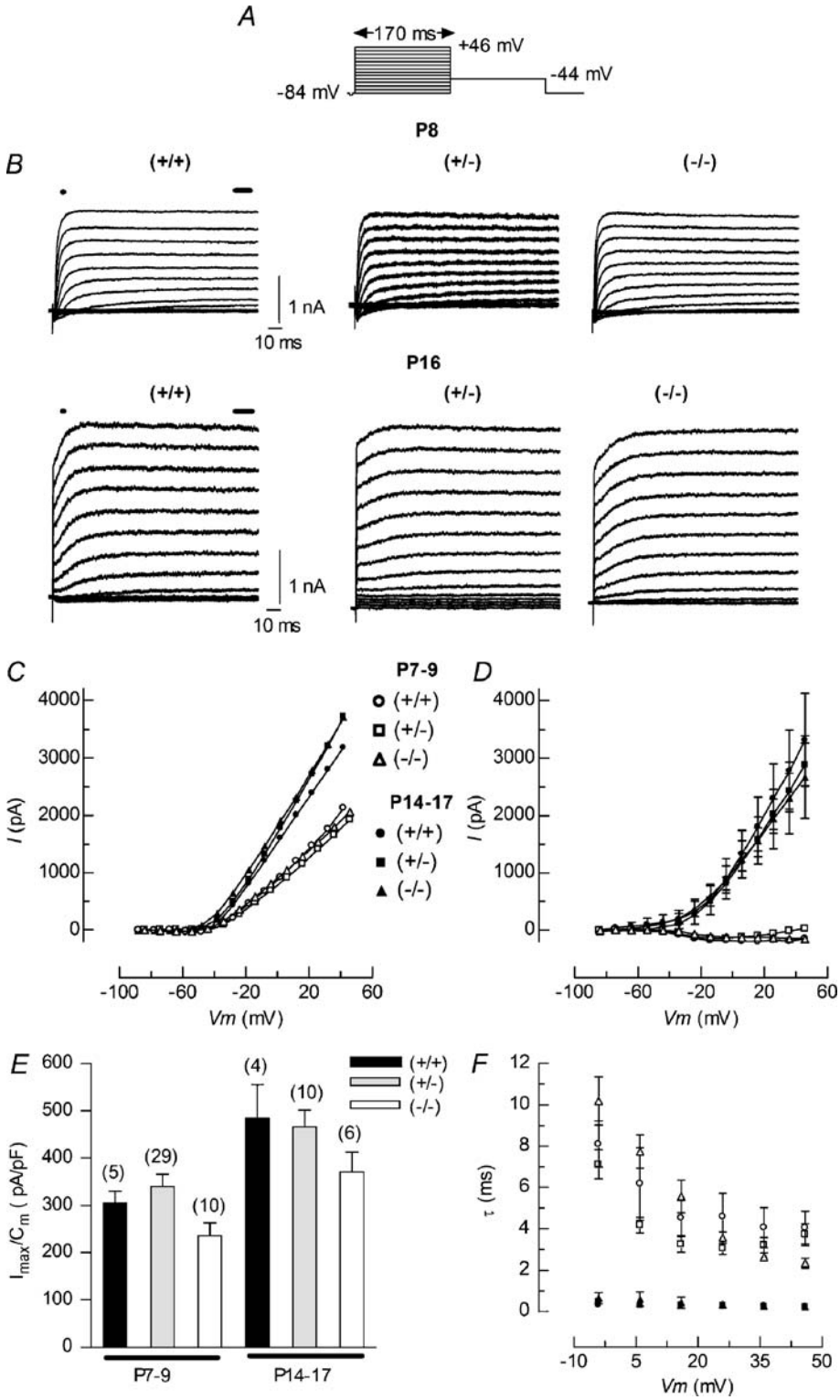
The inward rectifier K^+ current (I_{K1}), like *Chrna10* and SK2, is transiently expressed in IHCs before the onset of hearing (Marcotti and Kros 1999; Katz et al. 2004). To evaluate if the lack of expression of the α10 subunit alters the expression of this inward rectifier K^+ current, we evaluated its onset during development. As illustrated in Figure 5, hyperpolarizing voltage steps between −54 and −154 mV (in 10-mV decrements), starting from a holding potential of −64 mV (Fig. 5A), elicited inward currents in P9 IHCs of *Chrna10*$^{-/-}$ mice and their *Chrna10*$^{+/+}$ littermates (Fig. 5B). Identical results were found in IHCs from P7–9 *Chrna10*$^{+/-}$ mice (not illustrated). The peak and steady-state I–V curves (Fig. 5C, D, respectively) show that the current became inward negative to −80 mV in both genotypes, near the K^+ equilibrium potential.

DISCUSSION

In this work, we studied the membrane electrical properties of developing cochlear IHCs in *Chrna10*$^{-/-}$ mice before and after the onset of hearing. We found that neither the spiking activity in IHCs nor the developmental functional expression of voltage-gated and/or calcium-sensitive K^+ channels in these cells is altered by the absence of the α10 nAChR subunit.

In rodents, cholinergic sensitivity of IHCs is known to peak around postnatal days 7–10 and to disappear after the onset of hearing (Katz et al. 2004). These changes are accompanied by a down-regulation of the expression of two key postsynaptic proteins, the α10 nAChR subunit (Elgoyhen et al. 2001; Simmons 2002; Katz et al. 2004) and the SK2 calcium-sensitive K^+ channels (Katz et al. 2004) as well as by the retraction of direct efferent synapses onto IHCs (Liberman et al. 1990; Simmons 2002). The key factors that signal the formation and the dismantling of this transient synapse are still unknown. We have previously shown that the lack of either the α9 or the α10 nAChR subunit disrupts the efferent inhibition on the gain of the cochlear amplifier and has a severe impact both on the morphology of the efferent-OHC synapse and in the sensitivity of cochlear hair cells to ACh (Vetter et al. 1999, 2007). Here, we show that the lack of the α10 subunit does not interfere with the normal functional expression and down-regulation of SK2 channels in IHCs (Katz et al. 2004). On the contrary, the genetic deletion of the functionally coupled SK2 channel has an extremely severe impact on the cholinergic efferent innervation (Murthy et al. 2009; Kong et al. 2008) in the cholinergic sensitivity of cochlear hair cells (Kong et al. 2008) and in the normal spiking activity of pre-hearing IHCs (Johnson et al. 2007). SK2 knockout mice lose the olivocochlear fibers and synapses after P18 (Murthy et al. 2009). In addition, both IHCs and OHCs fail to respond to either synaptically released or exogenously applied ACh even though the mRNA levels for both the α9 and the α10 nAChR subunits are similar to those found in wild-type mice (Kong et al. 2008). So it seems that SK2 channels are necessary to trigger the steps leading to the functional localization of the α9α10 nAChR at the plasma membrane of IHCs and OHCs. The present results, however, demonstrate that in spite of the concerted regulation of the expression of the *Chrna10* and the SK2 genes during development, the α10 nAChR subunit is not necessary for the functional expression of the SK2 channel in IHCs.

The present results also show that the lack of the α10 subunit does not cause major alterations either in the functional expression of other voltage-gated and/or calcium-sensitive K^+ channels or in the electrical properties of IHCs. Even though spiking activity is



present in the SK2 mutants, this spiking is not sustained (Johnson et al. 2007), as SK2 is necessary for the fast repolarization of the calcium action potentials (Marcotti et al. 2004). Moreover, it was shown that the lack of SK2 channels alters the normal calcium dependence of transmitter release after the onset of hearing (Johnson et al. 2007). The activity of the transient efferent synapse was shown to control

FIG. 3. Outward K^+ currents in mouse cochlear IHCs during development. **A** Pulse protocol used to determine the total outward K^+ currents. Voltage-dependent currents were obtained by holding the cell at −84 mV and stepping the voltage to 46 mV in 10-mV increments (170-ms duration). **B** Representative outward currents in IHCs from *Chrna10*$^{+/+}$, *Chrna10*$^{+/-}$, and *Chrna10*$^{-/-}$ mice prior (*top records*) or after (*bottom records*) the onset of hearing. Outward currents recorded from IHCs before the onset of hearing (P8) activate slowly in the three genotypes. In contrast, outward currents recorded from IHCs after the onset of hearing (P16) show a rapidly activating component. **C** Steady-state current–voltage curves of the currents shown in **B** measured at the long markers (amplitude values obtained between 170 and 182 ms were averaged) for the three genotypes. **D** Averaged current–voltage curves of the fast component corresponding to $I_{K,f}$ determined at the time of the short markers (only one point was computed) shown in **B** for the three genotypes. **E** Bar diagram illustrating averaged amplitudes at 46 mV normalized to the mean capacitance (C_m) in each age group for the three genotypes. For the three genotypes, amplitudes were significantly larger in the P14–17 age group compared to the P7–9 one (p values were 0.01, 0.02, and 0.03 for *Chrna10*$^{+/+}$, *Chrna10*$^{+/-}$, and *Chrna10*$^{-/-}$, respectively). **F** Time constants of activation (τ) for these currents are compared over a range of positive command potentials in the three genotypes. Mean±SEM in **D–F** were obtained in five, 29, and ten P7–9 IHCs from *Chrna10*$^{+/+}$, *Chrna10*$^{+/-}$, and *Chrna10*$^{-/-}$, respectively, and in four, ten, and six P14–17 IHCs from *Chrna10*$^{+/+}$, *Chrna10*$^{+/-}$, and *Chrna10*$^{-/-}$, respectively.

the firing frequency of calcium action potentials in IHCs (Glowatzki and Fuchs 2000; Goutman et al. 2005). In addition, these Ca^{2+} spikes trigger transmitter release from the ribbon synapses before the onset of hearing (Beutner and Moser 2001; Johnson et al. 2007). Therefore, even though in the present work we did not test transmitter release from IHCs, we cannot disregard the possibility that the genetic ablation of the α10 nAChR subunit could alter transmitter release from IHC ribbon synapses either before and/or after the onset of hearing.

In agreement with our results, however, the genetic ablation of SK2 channels does not have any major effects on the electrical properties and on the functional expression of K^+ ion channels during development (Johnson et al. 2007; Kong et al. 2008). These findings with α10 and SK2 knockout mice are surprising, since during development, IHCs undergo major and finely orchestrated changes in the expression of K^+ (Kros et al. 1998; Marcotti et al. 2003a) and Ca^{2+} channels (Beutner and Moser 2001), synaptic proteins (Eybalin et al. 2002), spiking activity (Glowatzki and Fuchs 2000; Beutner and Moser 2001; Marcotti et al. 2003a, 2004) and also in their cholinergic sensitivity and pattern of efferent innervation (Liberman et al. 1990; Simmons 2002; Katz et al. 2004). Before the onset of hearing, Ca^{2+} spikes are driven by the concerted activity of voltage-gated outwardly rectifying K^+ channels, fast inwardly rectifying K^+ channels, and voltage-gated Na^+ and Ca^{2+} channels (Eatock and Hurley 2003; Marcotti et al. 2003b; Housley et al. 2006). After the onset of

TABLE 3

Activation rates (τ) and reversal potential (V_r) of voltage-gated outward currents

	Chrna10									
	(+/+)		(+/−)			(−/−)			*ANOVA (p value)*	
Age	τ *(ms)*	V_r *(mV)*	τ *(ms)*	V_r *(mV)*		τ *(ms)*	V_r *(mV)*		τ	V_r
P7–9	4.05±0.8 (*n*=6)	−71.7±3.6 (*n*=4)	3.7±0.5 (*n*=12)	−68.8±1.5 (*n*=11)		2.4±0.2 (*n*=12)	−67.0±3.8 (*n*=3)		<0.05	0.52
P14–17	0.25±0.02 (*n*=2)	−67.5±3.3 (*n*=4)	0.22±0.03 (*n*=11)	−67.3±1.8 (*n*=12)		0.23±0. 07 (*n*=4)	−72.6±5.1 (*n*=4)		0.84	0.45

Time constants of activation (τ) were calculated by fitting a mono-exponential equation to currents elicited by a voltage step from a V_{Hold} of −84 to 46 mV. Reversal potentials (V_r) of total outward currents were determined by applying a 170-ms conditioning pulse of −4 mV, followed by a series of test pulses from −114 to −14 mV. The statistics shown in the table correspond to an ANOVA between data obtained in IHCs from *Chrna10*$^{+/+}$, *Chrna10*$^{+/-}$, *Chrna10*$^{-/-}$ mice. For the three gentotypes, activation rates were faster at P14–17 than at P7–9 (p<0.03). No differences were found in V_r in any of the three genotypes between P7–9 and P14–17 (p>0.5)

n number of IHCs tested

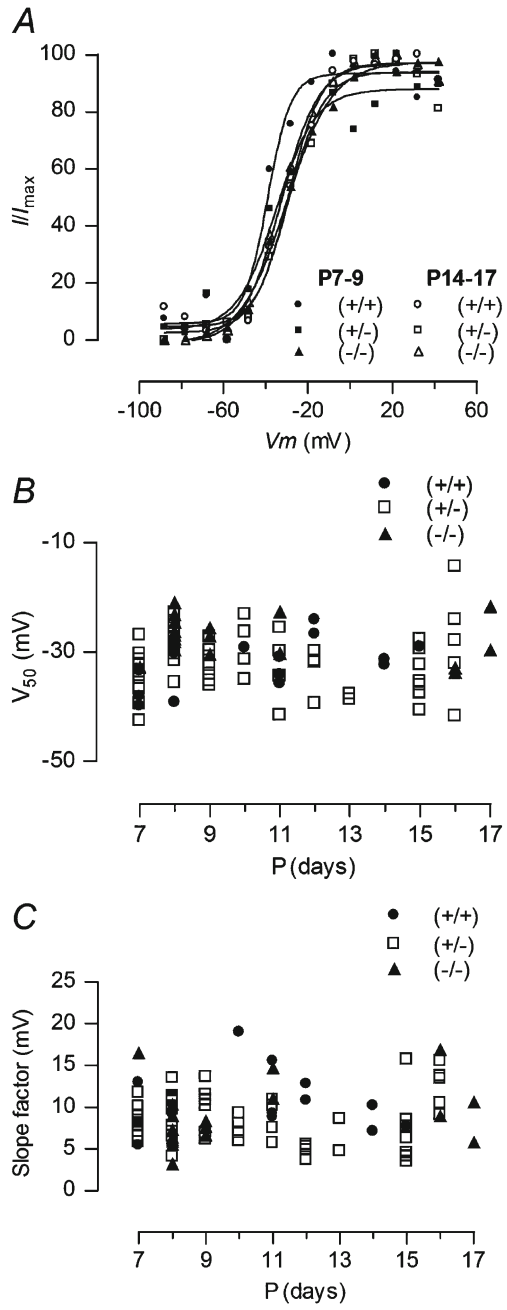


FIG. 4. Activation of outward K^+ currents during IHC maturation. **A** Typical examples of normalized steady-state activation curves for total outward currents in IHCs from *Chrna10*$^{+/+}$, *Chrna10*$^{+/-}$, and *Chrna10*$^{-/-}$ mice prior (P7–9) or after the onset of hearing (P14–17). Activation curves were obtained by analyzing tail currents at −44 mV after a series of conditioning depolarizing steps (170 ms in duration) in 10-mV increments from −84 mV and then plotting the normalized instantaneous tail currents (measured 0.2–0.4 ms after stepping to the test potential) against the different pre-pulse potentials. Data were fitted by a single first-order Boltzmann equation (n=2–12 IHCs per genotype). **B** Potential of half-maximal activation (V_{50}) for the activation curves of total K^+ currents recorded in IHCs from P3 to P17 in the three genotypes. **C** Slope factor (S) for the activation curves of total K^+ currents recorded in IHCs from P3 to P17 in the three genotypes (n=2–12 IHCs per genotype).

hearing, IHC spiking disappears probably because Na^+, Ca^{2+}, and fast inwardly rectifying channels become less numerous, whereas outwardly rectifying K^+ channels increase in number (Eatock and Hurley 2003; Marcotti et al. 2003b). The correct developmental functional maturation of IHCs has been shown to be highly sensitive to alterations in the expression of some of these proteins. For example, mice lacking $Ca_v1.3$ channels show not only alterations in afferent synaptic transmission and spiking activity but also in the expression of large-conductance Ca^{2+}-activated K^+ channels (BK channels; Brandt et al. 2003). In addition, the normal developmental pattern of cholinergic sensitivity, efferent innervation, and SK2 channel expression are altered in $Ca_{v1.3}$ knockout mice. Thus, contrary to the cessation of ACh sensitivity normally seen in wild-type mice at around P13-14 (Katz et al. 2004), it persists up to 6 weeks after birth in $Ca_{v1.3}$ knockout mice (Brandt et al. 2003; Nemzou et al. 2006). Moreover, during this critical developmental window, several studies have shown that modifications in the expression of proteins involved in thyroid hormone signaling alter IHC spiking activity, probably due to the lack of expression of BK and KCNQ4 channels together with an increase in calcium influx (Eatock et al. 1998; Brandt et al. 2007; Sendin et al. 2007). These data indicate that there is a concerted regulation of the functional expression of ion channels during IHC maturation. Therefore, we expected that the lack of the α10 nAChR subunit, and thus the absence of a functional nAChR receptor in IHCs (Vetter et al. 2007), would interfere with the normal pattern of expression of other ion channels. Notwithstanding, this was not the case as shown by the normal development of the electrophysiological properties of developing IHCs from *Chrna10*$^{-/-}$ mice.

The spiking activity (threshold, generation, and frequency) of neonatal IHCs of *Chrna10*$^{-/-}$ mice was identical to those of their wild-type and heterozygous littermates. Moreover, the amplitude and temporal course of the Ca^{2+} spikes were not altered by the lack of the α10 subunit. These results are in line with the lack of significant changes in the functional expression of K^+ ionic channels found in the present work. They also strongly suggest that the developmental expression of both Ca^{2+} and Na^+ channels is normal in IHCs from *Chrna*$^{-/-}$ mice (Marcotti et al. 2003a, 2004; Housley et al. 2006). As mentioned above, both the generation and the frequency of this spiking activity in IHCs are regulated by the transient inhibitory efferent cholinergic innervation from the olivocohlear system (Glowatzki and Fuchs 2000; Goutman et al. 2005) that is mediated by the postsynaptic α9α10 nAChR (Elgoyhen et al. 1994, 2001; Glowatzki and Fuchs 2000; Katz et al. 2004; Marcotti et al. 2004; Gomez-Casati et al. 2005). As expected, due to the lack of cholinergic sensitivity of

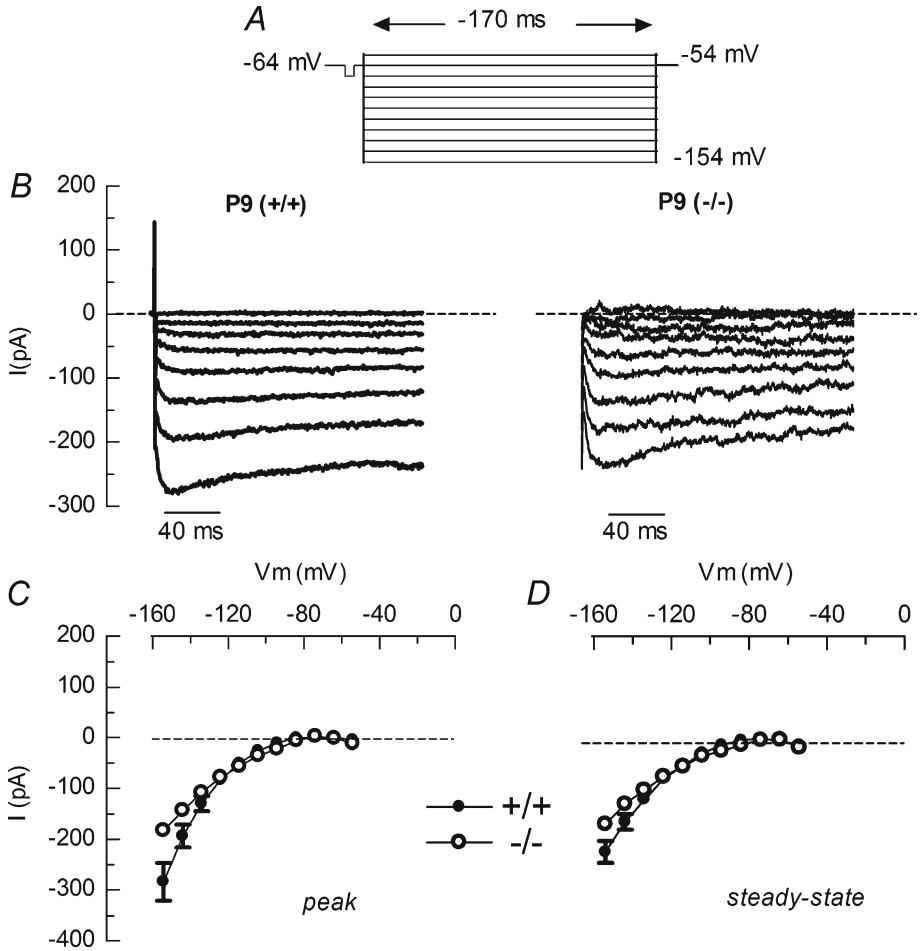


FIG. 5. Inwardly rectifying K^+ current in cochlear IHCs. **A** Protocol used to measure inwardly rectifying K^+ currents in IHCs. These currents were obtained by using 10-mV voltage steps between −24 and −154 mV starting from a holding potential of −64 mV. **B** Typical current responses in IHCs from *Chrna10*$^{+/+}$ and *Chrna10*$^{-/-}$ P9 mice. **C** Peak current–voltage curves from *Chrna10*$^{+/+}$ (*filled circles*) and *Chrna10*$^{-/-}$ (*clear circles*; *n*=4 IHCs per genotype). **D** Steady-state current–voltage curves from *Chrna10*$^{+/+}$ (*filled circles*) and *Chrna10*$^{-/-}$ (*clear circles*; *n*=8 IHCs per genotype).

IHCs from *Chrna10*$^{-/-}$ mice (Vetter et al. 2007), neither the generation of calcium action potentials nor their frequency was sensitive to ACh.

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