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Responses of Pigeon Vestibular Hair Cells to Cholinergic Agonists and Antagonists

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Abstract

Acetylcholine (ACh) is the major neurotransmitter released from vestibular efferent terminals onto hair cells and afferents. Previous studies indicate that the two classes of acetylcholine receptors, nicotinic (nAChRs) and muscarinic receptors (mAChRs), are expressed by vestibular hair cells (VHCs). To identify if both classes of receptors are present in VHCs, whole cell, voltage-clamp- and current-clamp- patch recordings were performed on isolated pigeon vestibular type I and type II HCs during the application of the cholinergic agonists, acetylcholine and carbachol, and the cholinergic antagonists, d-tubocurarine and atropine. By applying in different combinations, these compounds were used to selectively activate either nAChRs or mAChRs. The effects of nAChR and mAChR activation on HC currents and zero electrode current potential (V_z) were monitored. It was found that presumed mAChR activation decreased both inward and outward currents in both type I and type II HCs, resulting in either a depolarization or hyperpolarization. Conversely, nAChR activation mainly increased both inward and outward currents in type II HCs, resulting in a hyperpolarization of their V_z . nAChR activation also increased outward currents in type I HCs resulting in either a depolarization or hyperpolarization of their V_z . The decrease of inward and outward currents and the depolarization of the V_z in type I pigeon HCs by activation of mAChRs represents a new finding. Ion channel candidates in pigeon vestibular HCs that might underlie the modulation of the macroscopic ionic currents and V_z by different AChR activation are discussed.

Keywords

pigeon; acetylcholine; muscarinic receptor; nicotinic receptor; vestibular; hair cells

1. Introduction

Vertebrate vestibular organs possess two kinds of hair cells so called type I and type II. Birds, mammals and reptiles (amniotes) have both type I and type II HCs whereas fish, frogs and other anamniotes only express type II HCs (Yan et al., 1991; Lysakowski, 1996). Pigeon and gerbil type I and type II hair cells can be distinguished by their shape, innervation and several of their ionic currents (Correia and Lang, 1990; Rennie and Correia, 1994; Ricci et al., 1997a). Similar observations have been made in turtle (Brichta and Goldberg, 2000;

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Brichta et al., 2002). Pigeon vestibular hair cells receive both the pre-synaptic (efferent) and post-synaptic (afferent) innervation. Afferent bouton nerve terminals innervate pigeon type II hair cells; whereas nerve calyces surround type I hair cells (Li et al., 2007; Correia et al., 1985). Pigeon efferent fibers originate in the reticular formation and brainstem (Eden and Correia, 1982; Schwarz et al., 1981) and course peripherally to innervate type I and type II hair cells, afferent calyces of type I hair cells and bouton afferents of type II hair cells (Li et al., 2007). Therefore, it is possible that efferent activation modulates the hair cell-afferent response both presynaptically (on HCs) and postsynaptically (on afferent terminals). In different animal models, it has been shown that efferent activation can either increase and/or decrease afferent firing activity (Dickman and Correia, 1993; Furukawa, 1981; Dechesne and Sans, 1980; Goldberg and Fernandez, 1980; Hartmann and Klinke, 1980; Holt et al., 2006; Rossi et al., 1980). Thus, the predominant efferent neurotransmitter, acetylcholine (ACh) may act on different types of acetylcholine receptors present on both HCs and afferent terminals to produce the different changes in vestibular afferent firing rates.

Two types of nAChRs are present on auditory and vestibular hair cells. One is defined by the association of the $\alpha 9$ and $\alpha 10$ nAChR subunits. It is selectively blocked by strychnine, tropisetron and α -bungarotoxin. The other nAChR is defined by its sensitivity to the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) (Holt et al., 2003). Activation of $\alpha 9/\alpha 10$ nAChRs in mammalian auditory hair cells (Elgoyhen et al., 1994; Elgoyhen et al., 2001) hyperpolarizes the hair cell membrane through the downstream activation of SK- I_{KCa} currents (Yuhua and Fuchs, 1999) on the hair cell's membrane which subsequently inhibits afferent firing (Art et al., 1982). On the other hand, the activation of the DMPP-sensitive nAChR in frog produces a rapid depolarization of semicircular canal (SCC) hair cells, probably through the classical nicotinic receptor action (Holt et al., 2003).

The details of activation of cholinergic muscarinic receptors are less well worked out. Indirect evidence suggests that mAChR activation may involve different transduction pathways leading to depolarization or hyperpolarization of frog type II VHCs (Guth and Norris, 1996). The hyperpolarization pathway is believed to incorporate the phosphatidylinositol pathway ultimately resulting in the activation of I_{KCa} currents (Shigemoto and Ohmori, 1991; Yoshida et al., 1994; Guth and Norris, 1996). I_{KCa} currents are present in pigeon type I (Rennie and Correia, 1994) and type II (Lang and Correia, 1989) VHCs. The depolarization pathway and the effector are still unknown. However, it is widely recognized that activation of mAChRs modulates a number of ionic currents directly or indirectly resulting in membrane depolarization in a variety of tissues (Caulfield, 1993; Inoue and Yoshii, 1992; Jones, 1993). Five strong candidate currents include a potassium M (muscarinic) – current, I_M , (Adams et al., 1982; Brown and Adams, 1980) an outwardly rectifying delayed rectifier current, I_{KD} (Harvey and Hume, 1989; Janssen, 1996), an inwardly rectifying potassium current I_{IRK1} (Jones, 1993; Jones, 1996; Jones, 1997; Jones, 2003; Rossignol and Jones, 2006), a L-type I_{Ca} (Fuchs and Murrow, 1992; Guth and Norris, 1996; Holt et al., 2003; Pemberton and Jones, 1997; Wikstrom et al., 1998) and a calcium-activated potassium current, I_{KCa} , (Kong et al., 2005). A variant of I_M , (I_{K1}), and I_{KCa} are present on pigeon type I hair cells (Rennie and Correia, 1994; Rennie et al., 2001). I_{IRK1} , I_{KCa} , L-type I_{Ca} and I_{KD} are expressed on pigeon type II VHCs (Correia et al., 2004; Masetto and Correia, 1997; Zampini et al., 2008; Lang and Correia, 1989).

The purpose of the present investigation was threefold. (1) To determine if there are functional cholinergic receptors on pigeon type I and type II vestibular hair cells. (2) To determine if the application of cholinergic agonists produces hyperpolarization and/or depolarization of type I and type II VHCs and (3) To try to parse mAChR activation from nAChR activation in type I and type II hair cells by monitoring changes in membrane

currents and voltages in VHCs while using a combination of cholinergic agonists and antagonists.

2. Results

2.1. Pharmacological studies of type II VHCs

During both ruptured voltage clamp and current clamp recordings described below, the total number of type II HCs activated by acetylcholine was 22.1% (38/172).

2.1.1. Voltage clamp responses during voltage step protocol—During the voltage step protocol (Figure 1A), the application of 100 μ M ACh increases outward currents (●) at voltage potentials of -60 mV and $+40$ mV and inward current (●) at -120 mV (Figure 1A) relative to control currents (□). Conversely, application of a mixture of tubocurarine (TC) with ACh decreases both inward (●) and outward (●) currents (Figure 1B). Under both pharmacological conditions, both outward and inward currents (▲) return towards control values after drug washout with normal external (NE) solution (Figure 1C). Since 10 μ M TC has been shown to block DMPP (Holt et al., 2003) and $\alpha 9/\alpha 10$ nAChRs (Holt et al., 2001; Elgoyhen et al., 2001; Verbitsky et al., 2000), we assume that the current (●) in Figure 1B results from the activation of mAChRs whereas that in Figure 1A results from activation of nAChRs. The above conclusion assumes that both nAChRs and mAChRs are simultaneously expressed on the same HC and that TC selectively antagonizes nAChRs.

Not all type II HCs had the same response to ACh application. Among 10 type II HCs that responded to ACh applications, 8 showed an increase in outward current (520.3 ± 399 pA with the range from 200 pA to 1400 pA) at the $+40$ mV potential while 7 showed an increase of the inward current (125.7 ± 32.3 pA with the range from 70 pA to 170 pA) at the -120 mV potential. Other HCs demonstrated a decrease of the outward current ($n=2$, 165.6 ± 31.1 pA) and a decrease of the inward current ($n=1$, 19.8 pA). Of the 6 HCs that were treated with the mixture of ACh and TC (nAChR blockage/mAChR activation), the outward current was either decreased (3/6 cells, 375.4 ± 478.9 pA with the range from 80 pA to 900 pA) or increased (3/6 cells, 423.9 ± 456.5 pA with the range from 30 pA to 900 pA) at $+40$ mV. The inward current was either decreased (2/6 cells, 132.9 ± 170.8 pA with the range between 10 pA to 250 pA) or increased (1/6 cells, 366.1 pA) when the voltage was held at -120 mV. The reason for these different responses of type II VHCs to the same drug application paradigm is not clear but it is possible that in some cases incomplete blockage of nAChRs occurred. Also, it may be due to the expression of different AChR subtypes observed on pigeon hair cells (Li et al., 2007) and/or the possibility that different channels carrying different cadres of ionic currents (e.g., I_{KCa} , I_{Kl} , $I_{Kir2.1}$, or I_{Ca}) (Weng and Correia, 1999) were expressed and modulated on different VHCs.

2.1.2. Voltage clamp responses during voltage ramp protocol—Ionic current modulation by drug application over the range of voltage potentials from -120 mV to $+40$ mV, using the voltage ramp protocol (Inset Figure 2A) was more obvious than that observed using the step protocol. The application of 100 μ M ACh to a type II HC increased the inward current (●) when the membrane potential was held more negative than the reversal potential (change in net current direction) and increased the outward current (●) when the voltage potential was more positive than the reversal potential (Figure 2A). Following wash of the HC with NE, the current was restored to control levels (▲). Subsequent administration (Figure 2B) of a mixture of 100 μ M ACh and 10 μ M TC to the same cell inhibited the inward (●) and outward currents relative to the control current (□). This effect is more clearly shown in Figure 2D (●). However, a second application of ACh again increased the inward and outward (●) currents on either side of the reversal potential (Figure 2C). Figure

2B suggests that TC antagonized nAChRs. ACh application produced a current reversal at -97 mV and the current ($\square$) reached the peak amplitude around -20 mV (Figure 2D). ACh + TC application produced change in inward and outward current trajectories ($\bullet$) because of nAChR blockage and mAChR activation (Figure 1D). In the absence of drug application (control and recovery) in Figures 2A-C and during ACh + TC application (Figures 2B), the reversal potential was around -60 mV. However, ACh application (Figures 2A, C), produced a shift of the membrane reversal potential from -50 mV to -80 mV (1st application) and to -73 mV (2nd application). Consistent with I/V plots in earlier studies (Lang and Correia, 1989), this nAChR-induced current (with its N-shaped I/V plot) is most likely carried by a calcium-activated potassium (SK) channel (Lang and Correia, 1989; Holt et al., 2003).

A total of 13 HCs had responses to ACh during the ramp protocol. Like the VHC that produced the traces shown in Figure 2A, 9/13 (69.2%) HCs showed an increase in the inward current (58.6 ± 47.8 pA at -120 mV with the range from 15 pA to 140 pA) and an increase in the outward current (182.6 ± 189.2 pA at $+40$ mV with the range from 50 pA to 550 pA). Of these 9 HCs, the increase was consistent over the entire voltage range. However, in 4/13 (30.8%) VHCs, the amplitude of outward current was decreased (388.6 ± 393.4 pA at $+40$ mV with the range from 80 pA to 900 pA) when the membrane potential was more positive than 6.4 ± 4.6 mV. However, 7/8 (87.5%) of the type II VHCs that changed current during application of a mixture of 100 μ M ACh and 10 μ M TC (assumed to result in mAChR activation) produced traces with a decreased amplitude of inward currents (51.3 ± 21.4 pA at -120 mV with the range from 30 pA to 80 pA) and decreased amplitudes of outward currents (108.4 ± 88.4 pA at $+40$ mV with the range from 80 pA to 280 pA).

The amplitudes of the currents during the ramps were smaller than those measured during the step protocols. This probably resulted because the current amplitudes were measured at different time points. During the ramps, the currents were measured at instantaneous voltage levels but during the steps, the currents measured were at steady state voltage levels. Other than the amplitude differences, the responses of type II VHCs to ACh and ACh with TC application did not differ between the voltage step and voltage ramp protocols.

Generally, cells treated with ACh were most likely to have an increase of inward (69.6%, 16/23) and outward (73.9%, 17/23) current; whereas cells treated with ACh and TC had a decrease of inward (64.3%, 9/14) and outward (71.4%, 10/14) current.

2.1.3. Current clamp responses involving the zero electrode current potential (V_z)—Application of 100 μ M ACh to type II HCs hyperpolarized the cell's V_z (Figure 3A). Mixtures of 100 μ M ACh plus 100 μ M atropine plus 10 μ M TC and of 100 μ M ACh with 10 μ M TC completely blocked this ACh-induced hyperpolarization in the same cell (Figure 3B). This suggests that the hyperpolarized response in Figure 3B was mainly through activation of nAChRs.

Over the range of membrane potentials measured in this study, (-61 mV $< V_m < -48$ mV) no depolarized response of type II VHCs hair cells to nAChR activation was observed.

Treatment of type II VHCs with of a mixture of ACh with TC (mAChR activation) produced either a hyperpolarization (Figure 3C) or depolarization (Figure 3D) of V_z . Similar responses were observed when a mixture of 2 or 100 μ M CCh and 10 μ M TC was applied (Figure 3E-F). The presence of a non-antagonized response by TC application implies that the responses in Figures (D-F) were through the mAChRs. Moreover, the fact that the hyperpolarized response to 2 μ M CCh and 10 μ M TC was blocked subsequently in the same trace by 2 μ M CCh + 10 μ M TC + 100 μ M atropine (Figure 3F), further confirms that

mAChRs were activated. Thus, for the range of responses presented in Figure 3, agonization of nAChRs produced hyperpolarization of V_z (Figure 3B) while agonization of mAChRs produced hyperpolarization or depolarization of V_z (Figures 3C-F).

A total of 57 VHCs produced usable current clamp recordings. The results from these recordings are summarized in Table 1. The number of VHCs whose V_z s were depolarized during mAChR activation treatment was 6 (11%, 18.3 ± 8.2 mV) and none of these were antagonized by 10 μ M TC application. The total number of HCs whose V_z s hyperpolarized following application of ACh or CCh was 15 (26 %, 10.1 ± 3.5 mV) and 6 of them produced ACh responses (40%, 10.6 ± 3.0 mV) that were blocked by the addition of TC. Of these six, two (33%) were also antagonized by 100 μ M atropine application. Traces from the remaining 9 cells, that produced a hyperpolarized V_z (9.8 ± 4.0 mV), that was not blocked by 100 μ M ACh + 10 μ M TC, were presumed result from mAChR agonization.

2.2. Pharmacological studies of type I VHCs

Ruptured patch recordings—Pigeon Type I HCs have larger outward currents and more negative V_z s than type II HCs (Suppl. Figures 1C-D and 2). These conclusions are consistent with previous results using different experimental conditions (Correia and Lang, 1990; Rennie and Correia, 1994). In addition, at $V_h = -60$ mV, type I HCs demonstrate a rapidly activating-slowly inactivating current, I_{KL} , (Supplementary Data Figure 1A) which is not present in type II HCs (c.f., Supplementary Data Figure 1B). These macroscopic current features along with V_z and cell shape were used to differentiate type I and type II HCs during each experiment.

Application of 2 μ M CCh to a type I HC profoundly reduced the large outward current during a voltage step protocol. The resulting I/V plots are shown in Figure 4A. Relative to control ($\square$) at +20 mV, the amplitude of the outward current ($\bullet$) was decreased 87%; while at -140 mV the inward current is decreased 35%. In addition, the zero current potential moved from -68 mV to -55 mV. Partial recovery was observed after NE washout ($\blacktriangle$).

Responses from a total of 18 type I HCs were obtained and 2 out of 18 (11%) type I HCs responded to ACh application. Higher percentages (summarized below) were obtained from perforated patch recordings.

Perforated patch recordings—Voltage and current clamp responses were obtained from a different sample of 29 type I HCs using the same batch of dissociated HCs but using the perforated patch clamp technique. This method precludes the transfer of divalent ions (e.g., Ca^{2+}) from the cell into the recording pipette (Rae et al., 1991). Moreover, the method prevents dialysis of the intracellular milieu, a property that favors observation of mAChR responses.

In a hair cell, different from the one shown in Figure 4A, during perforated patch clamp recording and using a negative-positive voltage step protocol (insert in Figure 4B), the delivery of 100 μ M ACh to the cell resulted in an inhibition (75.4 %) of the steady state outward current ($\bullet$), compared to NE control condition ($\square$). Additional treatment of the HC with 100 μ M ACh + 10 μ M TC produced a further reduction in outward steady state current ($\circ$) of 27% relative to ACh alone. The steady state outward current ($\blacktriangle$) recovered to 67% of control level following NE wash (Figure 4B).

Three of 21 type I VHCs that showed increases in outward current following application of ACh or CCh. In Figure 4C it can be seen that outward steady state current during a step protocol increased relative to the control trace ($\square$) by 12 % following 0.6 s of superfusion ($\bullet$) with ACh and by 27% following an additional 0.6 s of ACh superfusion ($\blacktriangle$). The

changes resulted from sequential voltage steps during continual ACh exposure. Application of the non specific cholinergic agonist ACh to the cell probably activated both mAChRs and nAChRs. The increased inhibition of the outward current by mAChR activation (response to ACh + TC, Figure 4B) indicated that the nAChR response (during ACh application) on the outward current was excitatory. In other words, nAChR activation increased the outward current and it might be the case in Figure 4C).

During the perforated patch clamp recordings, reduction of the outward current in type I hair cells during cholinergic agonist application was the predominant response. In the sample of 21 type I hair cells 17/21 (81%) demonstrated a decrease in outward current following application of cholinergic agonists ACh and CCh while 3/21 (14%) showed an increase and 1 (5%) showed no change. The inward current at -120 mV (deactivation of current on at -60 mV, see (Rennie and Correia, 1994) also changed in some type I HCs during ACh application. Twelve of 21 (57%) HCs showed an increase; 3/21 (14%) HCs demonstrated a decrease in inward current and 8/21 (37%) showed no change. Moreover, in four type I HCs also treated with ACh + TC, the inward current showed decrease in 1/4, an increase on 1/4 and no change in 2/4.

Both V_z depolarization and hyperpolarization were observed in type I VHCs during perforated patch-current clamp recordings (Figure 4D, E). In Figure 4D, the depolarization (26.5 mV) induced by $100 \mu\text{M}$ ACh application was not blocked by the subsequent addition of $10 \mu\text{M}$ TC. As can be seen in Figure 4D, the “off” response to ACh application was longer in duration than the “on” response. Also, an overshoot was seen at the end of the off response during repolarization. During the first application of $100 \mu\text{M}$ ACh, the off/on duration ratio was 8. The off/on duration ratio was the same order of magnitude (13) for the shorter duration application of $100 \mu\text{M}$ ACh + $10 \mu\text{M}$ TC. In a different later test, the same cell was treated with $100 \mu\text{M}$ ACh + $10 \mu\text{M}$ TC for 40 s. During that test, the off/on duration ratio was 7. In Figure 4E, application of $10 \mu\text{M}$ TC + $200 \mu\text{M}$ CCh prevented V_z hyperpolarization resulting from application of $200 \mu\text{M}$ CCh alone. Application of $200 \mu\text{M}$ CCh produced a similar on and off response (off/on duration ratio = 1.1). The absence of blockage of the response during ACh + TC application suggests that the response in Figure 4D results from activation of mAChRs. The longer off duration suggests a metabotropic response. The blockage of the response by addition of $10 \mu\text{M}$ TC to $200 \mu\text{M}$ CCh and the symmetrical on-off response in the trace shown in Figure 4E suggests activation of nAChRs and an ionotropic response.

V_{zs} were measured for a group of 10 type I HCs during current clamp recording. As summarized in Table 1, ACh application produced a hyperpolarized response in 3 of the cells and a depolarized response in 7 of the cells. The magnitude of the depolarization was $8 \text{ mV} \leq \Delta V_z \leq 54 \text{ mV}$ with a mean $\pm$ S.D. (N) of $24 \pm 16 \text{ mV}$ (N = 7). The magnitude of the hyperpolarization was $11 \text{ mV} \leq \Delta V_z \leq 26 \text{ mV}$ with a mean $\pm$ S.D (N) of $19 \pm 6 \text{ mV}$ (N = 3). In 6/10 cells, $100 \mu\text{M}$ ACh + $10 \mu\text{M}$ TC was given to the same cells during either voltage clamp or current clamp recordings. This treatment did not block depolarized responses of 2 cells (e.g., Figure 4D) but blocked the hyperpolarized response in 1 cell (e.g., Figure 4E). The blockage of the V_z responses following application of ACh plus TC was presumably due to nAChR antagonism whereas the lack of blockage was presumed to be due to mAChR agonism.

Perforated patch clamp recordings were made from a total of 77 type I HCs. Of these 29 (37%) responded to ACh/CCh and 48 (63%) did not. The threefold increase in percentage (37% vs. 11%) of type I hair cells responding to ACh and CCh during perforated patch versus ruptured patch experiments is probably due to the difference in experimental conditions (e.g., perforated versus ruptured patch, patch clamp versus voltage follower

amplifier or superfusion system - pressure driven vs. gravity driven). Most likely perforated patch recording which precluded intracellular content dialysis and large ion (e.g., Ca^{2+}) exchange made the difference. Generally, however, both ruptured and perforated patch current clamp data for type I VHCs, summarized in Table 1, indicated that V_z depolarization and hyperpolarization were induced by the activation of either nAChRs or mAChRs.

3. Discussion

In the present study we were able to show that functional AChRs were present on both type I and type II pigeon VHCs. We concluded that either nAChRs or mAChRs were activated in both type I and type II hair cells. We demonstrated, using AChR agonists and antagonists, that activation of mAChRs or nAChRs produced either increases or decreases in inward and outward ionic currents and hyperpolarization and depolarization of the HC's membrane potential. Table 1 summarizes these observations and shows that for type II hair cells, activation of nAChRs produces an increase in both inward and outward currents and a hyperpolarization of the V_z . The hyperpolarized V_z confirms earlier studies showing that ACh on short (outer) hair cells of the chicken basilar papilla, turtle cochlear HCs and frog isolated saccular HCs produces a hyperpolarization of the HC membrane potential (Fuchs and Murrow, 1992; Art et al., 1984; Holt et al., 2003). The N-shaped current traces in Figure 2D suggest that stimulation of nAChRs may result in activation of an outward I_{KCa} current carried by SK type channels resulting hair cell membrane hyperpolarization. This type of channel has been observed in pigeon type I hair cells (Rennie and Correia, 1994). Pigeon type II hair cells (Lang and Correia, 1989) also express I_{KCa} currents (type unknown). Apparently ACh responses can be carried by the SK or BK type since ACh-induced I_{KCa} current (through BK type channels) has been observed in guinea pig type II VHCs (Kong et al., 2007).

Activation of mAChRs in pigeon type II HCs inhibited both inward and outward currents which resulted in both hyperpolarized and depolarized V_z responses. These results are supported by previous studies. For example, a decrease in inward current (Jones, 1996; Firth and Jones, 2001) has been reported in tsA cells transfected with muscarinic receptor subtype M1 and the IRK1 (Kir2.1) ion channel. Also depolarization of the membrane potential of RBL-2H3 cells endogenously expressing IRK1 has been demonstrated (Jones, 1997). Finally, activation of M1, M3 or M5 transfected into NIH 373 cells reduced the I_{Ca} through G-protein activation of protein kinase C (PKC) (Pemberton and Jones, 1995; Pemberton and Jones, 1997). Pigeon and chicken vestibular type II hair cells demonstrate non-inactivating L-type Ca^{2+} currents (Lang and Correia, 1989; Zampini et al., 2006; Masetto et al., 2005) as well as inwardly rectifying IRK1 (Kir2.1) currents (Masetto and Correia, 1997; Zampini et al., 2008; Correia et al., 2004). Thus, pigeon type II hair cells have functional AChRs and the transduction machinery to modulate inward and outward currents of channels known to be in the pigeon VHC membrane. Moreover, the type II hair cell's membrane potential can be depolarized or hyperpolarized depending on the channel activated.

Activation of nAChRs in type I VHCs produced an increase in outward current and depolarization and hyperpolarization of the hair cell (Table 1). Activation of mAChRs in type I HCs produced a decrease in outward current and depolarization and hyperpolarization of the type I HC's V_z (Table 1). These previously unreported results are surprising since the pigeon type I hair cell, in vivo, is surrounded by a nerve calyx. Only rarely (~2%), do efferents synapse directly on a type I hair cell (Li et al., 2007), yet 11% (ruptured patch) and 36% (perforated patch) of type I hair cells, demonstrated decreases in inward and outward current resulting in V_z depolarization and hyperpolarization following mAChR or nAChR activation, (Table 1). The question arises; how does ACh released by efferent terminals cross the calyx to depolarize or hyperpolarize a type I hair cell?

In the case of mAChR activation, ~25% of pigeon type I HC calyces show abutting efferent (vesiculated) terminals and synaptic processes. M1, M2, M4 and M5 mAChR subtypes have been shown immunohistologically to be present in pigeon type I HC calyces (Li et al., 2007). Pigeon type II hair cells are immuno-reactive to all 5 mAChR subtypes (Li et al., 2007). Thus, there is evidence that pigeon type I HC calyces and type I and type II hair cells contain muscarinic cholinergic receptor subtypes. Cabadak et al., (2009) found that carbachol caused production of l-citrulline (a nitric oxide (NO) marker) in K562 erythroleukaemic cells. The effects were antagonized by atropine and 4-DAMP (M3-mAChR antagonists). Activation of mAChR increases amylase secretion in rat parotid gland cells. The secretion was inhibited (Rosignoli and Perez, 2002) by NO and nitric oxide synthase (NOS) inhibitors. NOS activity was noted in the apical plasma membranes of rat parotid glands after ACh application (Ishikawa et al., 2002). Thus, ACh activation of mAChRs has been shown to be related to NO and NOS production. NOS subtypes have been immune-labeled in vestibular and cochlear epithelia (Lysakowski and Singer, 2000) and specifically eNOS has been labeled in the type I hair cell calyx (Hess et al., 1998a; Hess et al., 1998b). Lv et al., (2010) using NO-selective electrodes measured a 3 fold release of NO in frog saccule neuroepithelium following application of ACh. In earlier studies, Chen and Eatock (2000) found that application of 4 NO donors inhibited the current $I_{K,L}$ in type I hair cells and patches of their membranes from 33 to 76%. The current $I_{K,L}$ is the dominant current in type I VHCs and the current studied in the present investigation. Chen and Eatock (2000) proposed that ACh, released from the efferents (due to the opening of Ca^{2+} channels in the efferent), crosses the efferent - calyx synapse and stimulates NOS to produce NO which diffuses across the calyx and inhibits $I_{K,L}$. No mention is made of the mechanism by which ACh stimulates NOS production or if there is another role that ACh plays in the process. Based on our present results and the more recent literature summarized above, we propose a mechanistic extension of the Chen-Eatock model. We suggest that in the 25% of the pigeon type I hair cells with a calyx-efferent synapse, the efferents release ACh that activates mAChRs in the calyx (Li et al., 2007) which causes the synthesis of NO by NOS in the calyx that diffuses across the calyx and inhibits $I_{K,L}$. Reduction of the $I_{K,L}$ outward current (Figure 4B) depolarizes the type I hair cell (Figure 4D) and increases the sensitivity of the cell. Additionally, ACh release from the efferent terminals abutting the apical-lateral surface pigeon type I hair cell membrane where the calyx does not cover the hair cell (Li et al., 2007) may activate mAChRs and inhibit the muscarinic - like current, $I_{K,I}$, directly.

$I_{K,I}$ ($I_{K,L}$) may be carried by a channel belonging to the KCNQ family of potassium channels. Kharkovets et al., (2000) have shown that KCNQ4 is expressed in mouse type I VHCs and calyces. It has been shown further that linopiridine, a KCNQ channel blocker, decreases $I_{K,I}$ in type I and $I_{K,D}$ in type II pigeon hair cells (Rennie et al., 2001). A variant of the KCNQ4 nucleotide sequence (Genbank accession # [GQ249112](#)) has been found in the pigeon brain. Recently, Holt et al., (2007) have provided strong evidence for KCNQ4 being the molecular substrate for the M-like current ($I_{K,I}$, $I_{K,L}$) in type I hair cells. It will be interesting to see if NO and/or ACh binding sites can be identified in the KCNQ4 sequence in future experiments.

Activation of nAChRs in type I HCs produced an increase in outward current and both hyperpolarization and depolarization of the type I HC's V_z (Table 1). This result can be partially explained by past studies that have shown that activation of the ionotropic nicotinic receptor in auditory and vestibular HCs containing $\alpha 9$ and $\alpha 10$ subunits (Elgoyhen et al., 1994; Elgoyhen et al., 2001) causes calcium induced calcium release from ER-like structures possibly residing in the post-synaptic cistern which activates SK- I_{KCa} currents and the resulting increase in outward current hyperpolarizes the HC's V_z (Kong et al., 2006; Glowatzki and Fuchs, 2000; Fuchs and Murrow, 1992; Housley and Ashmore, 1991; Yuhans and Fuchs, 1999; Holt et al., 2003). Pigeon type I hair cells demonstrate SK-

I_{KCa} currents (Rennie and Correia, 1994) but it has not been shown that they or their calyces express nicotinic nAChRs comprised of $\alpha 9/\alpha 10$ subunits. The depolarization could be due to the activation of DMPP-sensitive nAChR channels (Holt et al., 2003). The open question is – how does nAChR activation on the lateral surface of the calyx result in ion channel openings on the hair cell membrane? Future studies along the lines of those reported above for mAChRs will address that question.

In conclusion, we have demonstrated that pigeon type I and type II hair cells have functional cholinergic receptors. We have shown that activation of these receptors increase or decrease outward currents in pigeon type I and type II hair cells. We confirmed previous results that ACh activation of mAChRs and nAChRs modulates the current and $V_{1/2}$ s in type II HCs and presented new results that mAChRs and nAChRs are on type I hair cells. We have presented a model by which activation of mAChRs on the type I calyx lateral surface can cross the calyx and activate mAChRs on type I hair cells.

4. Experimental Procedures

4.1. Animals

Adult (6–40 wk old) white king pigeons (*Columbia livia*) (Double T Farms, Glenwood, IA) of either sex were used for the present studies. During and prior to surgery, the pigeons were anesthetized using an isoflurane system (VWR International, Cat. # 100229-042, West Chester, PA). Thirty minutes prior to anesthesia, the pigeons were given 0.01–0.02 mg/kg atropine (IV) to decrease bronchial and salivary secretions. Liquid isoflurane and O_2 were mixed in an isoflurane VIP 3000 vaporizer (Midmark, Cat. # 91305430, Versailles, OH). Flow of O_2 was set at 100–200 cc/min and the concentration of isoflurane was held at 4–5%. The output of the vaporizer was connected to either (switch selectable) a small feline mask or to a T-shaped “flow by” tube that could be connected to a tracheotomy tube. Excess isoflurane/ O_2 was vented to a self contained “trap”. Initially, the pigeon’s head was put into the feline mask and once anesthesia was achieved, a 9 cm tracheal tube was non-invasively inserted into pigeon’s trachea and the pigeon was placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Next, the T-shaped flow-by tube was connected to the tracheotomy tube. Flow was directed through the tube and the pigeon was observed. After the pigeon was completely unresponsive to foot pinch, the concentration of isoflurane was decreased to 2–3% for maintenance.

Under deep anesthesia, the labyrinths containing the vestibular end organs (including SCCs, utricle and saccule) were taken out from the bony labyrinths (Correia et al., 1989) and put in ice-chilled low Ca^{2+} dissociation solution to perform the isolation of single vestibular hair cells. The experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee at UTMB. Every effort was made to limit the number of animals used and the suffering of each animal.

4.2. Isolation of vestibular hair cells

The extracted end organs were dissected apart using fine iris scissors in 4°C low Ca^{2+} saline (containing in mM: 0.1 $CaCl_2$, 110 NaCl, 2 KCl, 2 $MgCl_2$, 3 D-Glucose and 10 HEPES at pH 7.25) (Hirano et al., 2004). Next, the end organs were incubated in 0.05% trypsin/EDTA (Cellgro, Cat. # MT25-052-CI, Manassas, VA) at room temperature for 6 min. To increase the efficiency of enzymatic activity on hair cells, the roof of the semicircular canals and the otolithic membranes of the utricle and saccule were removed prior to putting them into the enzyme solution. After the enzyme treatment, the dissected vestibular end organs were kept in 10% fetal bovine serum (FBS, Sigma, Cat. # F2442-500ML, St. Louis, MO) for 30 sec. Next the end organs were immersed in 500 $\mu g/\mu l$ bovine serum albumin (BSA, Fisher, Cat. #

03-600-501, Pittsburgh, PA) for 10 min. Both FBS and BSA were dissolved in the low Ca^{2+} solution that was freshly prepared on the day of the experiment. Next, a glass wisp was used to dissociate hair cells by gently stroking the vestibular neuroepithelium in 200 μl of a low Ca^{2+} solution. To separate the hair cells that were still attached to supporting cells, the hair cell/low Ca^{2+} solution was triturated in and out of a pipette having a fire polished 100 μm tip. Finally, the dissociated hair cells were put into a recording chamber whose cover slip bottom was coated with 0.5 mg/ml concanavalin A (Sigma, Cat. # 7275, St. Louis, MO). The cells were allowed to settle for 15 min before the NE superfusion was started. Concanavalin A helped the isolated hair cells attach to the recording chamber bottom without damage and prevented them from floating away during patch clamp and drug application.

To determine the optimal yield of isolated vestibular hair cells while keeping them in good condition, the hair cells treated with 0.05% trypsin/EDTA were compared with two other dissociation methods. The first was physical dissociation without enzyme treatment. All the steps described above were carried out within the low Ca^{2+} solution. It turned out that most of the hair cells were clustered or died. Low Ca^{2+} saline treatment (at least 10 min incubation) did not successfully break the tight junctions between hair cells and adjacent supporting cells. The yield of isolated single hair cells was poor. Furthermore, patch clamp of the few available isolated hair cells was difficult. It was hard to form a tight seal and cells died within a few seconds. The second method of hair cell dissociation involved treatment with a different enzyme. The vestibular end organs were initially treated with 65 $\mu\text{g}/\text{ml}$ protease XXIV. The yield of isolated hair cells after protease XXIV treatment was as good as that with trypsin/EGTA. The condition of the isolated hair cell membrane was smooth and easily formed a tight seal with the electrode. However, very few hair cells (3/50, 6%) had a response to ACh application. Protease XXIV enzymatic treatment of hair cells seemingly altered the AChRs or disrupted the intracellular signaling cascade and resulted in a majority of nonresponsive cells (Holt et al., 2001). Given the results of the above described trials on dissociation methods, hair cells, treated with 0.05% trypsin/EDTA, were used routinely.

The identification of type I and type II HCs was based on their morphology, V_z , and ionic current properties. Hair cells with little or no difference in base, neck and cuticular plate width were deemed as type II hair cells; while those having a small neck were considered to be type I hair cells (Ricci et al., 1997b). Additionally, the two types of HCs were separated based on V_z and their activation and inactivation properties. The average type I hair cell's V_z is 10 to 20 mV more negative on average than the V_z of a type II VHC. Moreover, type I hair cell currents (with the membrane potential held at -60 mV) activate more rapidly and inactivate more slowly than type II hair cells (c.f. Suppl. Figure 1(A) and Figure 1(B)).

4.3. Electrophysiology

The recording system for the ruptured patch electrophysiological studies, included: an Axopatch 200 amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA), a DigiData 1200 Interface (AD/DA converter, Axon Instruments/Molecular Devices, Sunnyvale, CA), a pressurized computer-controlled drug application system (model DAD-12, ALA Scientific Instruments, Westbury, NY) and a personal computer. The applied waveforms and acquired responses were controlled using the "Clampfit" suite of programs (Clampex V8, Axon Instruments/Molecular Devices, Sunnyvale, CA). Hair cells were viewed using a Nikon (Nikon Corp., Tokyo, Japan) upright microscope with fluorescent and differential interference contrast (DIC) illumination and an X40 water immersion lens. Hair cell images were magnified and viewed on a Sony video monitor (Sony Corp. of America, New York, New York). Attached nerves were not observed on the hair cells after our dissociation protocol. Additionally, only currents noted previously for hair cells (see **Introduction**) and

not calyces (Rennie and Streeter, 2006) were noted. The recording microelectrode position was controlled by a Burleigh PCS 1000 three dimensional piezoelectric micropositioner (Burleigh Instruments, Fishers Park, NY, USA). Electrodes were made from borosilicate blanks (0.75 ID, 1.50 OD, no. 1B150F-3, World Precision Instruments, Sarasota, FL, USA) containing a thin filament. The pipettes were pulled on a P-2000 puller (Sutter Instruments, Novato, CA, USA) and fire-polished on a MF-83 microforge (Narishige USA Inc., Greenvale, NY, USA). Electrode capacitance was greatly reduced by the following procedure which obviated further pipette coating. The glass blanks were initially washed with Chromerge (VWR International, Cat. # 21865-000, West Chester, PA, USA), silanized with 5% dimethyldichlorosilane (Sigma, Cat. # 40140, St. Louis, MO, USA) in chloroform (Fisher, Cat. # C298-500, Pittsburgh, PA, USA), then subsequently washed multiple times with double distilled water and dry heat sterilized at 240°C. The whole cell recording electrode impedance was typically 2–5 M Ω . Series resistance (R_s) and cell membrane capacitance (C_m) were measured directly from the amplifier's compensation dials. R_s was electrically compensated 90%. The average residual value was between 1–2 M Ω . The mean C_m of type I and type II vestibular hair cells was 16.9 ± 4.3 pF ($n = 25$) and 17.6 ± 3.1 pF ($n = 256$), respectively.

4.3.1. Voltage and current clamp protocols—In order to study AChR - induced modulation of the outward and inward currents in isolated vestibular hair cells, two voltage protocols were used. The first one was a negative to positive voltage step protocol. Initially, the voltage was held at -60 mV for 30 ms, then stepped to -120 mV for 230 ms, then stepped to $+40$ mV for 230 ms and finally stepped back to -60 mV. The second protocol was a negative to positive voltage ramp protocol. The membrane potential was held at -60 mV for 25 ms, then -120 mV for 25 ms, then ramped to $+40$ mV within 445 ms and finally returned to -60 mV for 5 ms. Each sweep in both protocols lasted 500 ms and there were 30 sweeps per run with a 2 s delay between each sweep. The protocol for type I HCs was different. The type I HCs were initially held at a voltage of -80 mV. The remainder of the protocol for type I HCs was the same as that for type II HCs. A third protocol was also used for current clamp studies. In that protocol, no current was injected into the cell but recordings were made of the membrane potential for 60 sec per sweep/run. During the duration of the recording, AChR agonists and antagonists were applied to the cell either by computer controlled pressure driven or gravity driven force. If the protocols varied they are specified in the figures or figure captions.

4.4. mAChR and nAChR agonists and antagonists

The agonists and antagonists used included: the general AChR agonists acetylcholine (Sigma, Cat. # A6625-25G, St. Louis, MO, 100 μ M) and carbachol (CCh, Sigma, Cat. # C4382-1G, St. Louis, MO, 2, 100 and 200 μ M), the selective nicotinic antagonist tubocurarine chloride hydrate (TC, Sigma, Cat. # T2379-100MG, St. Louis, MO, 10 μ M) and the muscarinic AChR antagonist atropine (Sigma, Cat. # A0132-1G, St. Louis, MO, 100 μ M). The drugs (1000X) were prepared as stock solutions in double distilled water (ACh, CCh and TC) or 100% ethanol (atropine). They were kept at -20°C and diluted into the NE solution at the desired concentration on the day of experiment. The concentration of TC was 10 μ M because previous studies showed that 1 μ M TC almost completely blocked the $\alpha 9$ nicotinic receptor that induced firing in primary afferent nerve fibers of the frog sacculle (Guth et al., 1994). Additionally, 10 μ M TC completely antagonized the DMPP-sensitive nicotinic ACh receptor in the frog posterior SCC (Holt et al., 2003) and the IC₅₀ for TC superfused onto oocytes with $\alpha 9$ injected into the cell was 0.3 μ M (Elgoyhen et al., 1994). The concentration of atropine was 100 μ M. Atropine in sub millimolar concentrations is considered to be a mAChR and nAChR blocker with an IC₅₀ in the nanomolar range (Richards, 1991; Gonzalez-Rubio et al., 2006). CCh at the concentration of 2 μ M is way

below the concentration to activate nAChR ($EC_{50} = 64 \mu\text{M}$ in cochlear HCs) (Verbitsky et al., 2000; Akk et al., 2005; Kitazawa et al., 2008). Therefore, we were able to test the effects of ACh on the composite family of mAChRs and nAChRs in both pigeon vestibular type I and type II hair cells.

Nicotinic and muscarinic receptors on type II and type I hair cells were differentiated by their response to drug mixtures. Hair cells that responded to ACh (CCh) but had blocked responses when ACh (CCh) + TC was used were considered to have their nAChRs activated. Hair cells that responded to ACh or CCh but showed no blocked response to ACh (CCh) + TC yet were blocked by ACh (CCh) + TC + atropine were considered to have mAChRs activated.

The drugs were superfused onto the cell mostly using a pressurized computer-controlled superfusion system. In other cases, a gravity controlled superfusion system was used. There appeared to be no difference in the magnitude or quality of the responses when using either system. The superfusion pipette (100 μm bore) was placed $\sim 30 \mu\text{m}$ from the cell; and the flow rate was $\sim 20 \mu\text{l/min}$. The recording chamber was continuously washed with NE in a rate of 1 ml/min. In voltage clamp mode, generally control recordings were initially collected for several sweeps (1–10) during the voltage protocol. Then, in subsequent groups of sweeps (11–20) drugs were applied. Finally during the last sweeps (21–30) and subsequent runs, if necessary, drugs were washed out by NE superfusion. Data that are presented in the figures, when indicated, are the average value of those sweeps during the drug application, control and recovery phases.

Control studies were carried out. To test if the responses were due to a “wave front” or pressure sensitive response, NE solution was superfused onto hair cells using several flow rates. No changes in V_z or currents were seen for the flow rates we used. Since internal Ca^{2+} is involved as a second messenger in pathways mediated by mAChR activation, EGTA and CaCl_2 were removed from the internal KCl pipette solution in some of the ruptured patch experiments. However, no qualitative differences were observed for the agonist/antagonist effects whether EGTA or CaCl_2 was in the pipette or not.

During the ruptured patch experiments, Na_2ATP (3 mM, Table 2) (Sigma, Cat. # A7699-1G, St. Louis, MO) was added to the pipette solution to: increase ATP-dependent enzyme activity and protein phosphorylation (Collins et al., 1987); maintain the requirement for GTP (Heidbuchel et al., 1990; Heidbuchel et al., 1993) and prevent run-down of Kir2.1 (Kamouchi et al., 1997).

Finally, instead of using MgSO_4 in the NE solution, MgCl_2 was used to maintain a balance of Cl^- in the internal and external solution. In control experiments, however, no differences were noticed between external solutions containing either MgSO_4 or MgCl_2 (Table 2).

4.5. Additional independent type I HC perforated patch clamp experiments

In a separate set of experiments, recordings were made from type I hair cells dissociated as described in section 4.2 but using the perforated patch technique (Horn and Marty, 1988; Rae et al., 1991) exclusively. The recordings were made using a different experimental “setup”. The recordings were made using an Axoclamp 2A (Axon Instruments) amplifier. Electrodes were pulled and polished to a tip diameter of 1.5–3.0 μm . The electrodes were back-filled with a solution containing a perforating agent. The perforating agent (Amphotericin B, Sigma, Cat. # A-4888, St. Louis, MO) was dissolved completely in dimethylsulfoxide (DMSO). Five milligrams of the perforating agent was dissolved in 50 μl of DMSO. This stock solution was diluted to a final concentration of 250 $\mu\text{g/ml}$ in the back filling solution. The components of the back filling solution are listed in Table 2. The pH of

the backfilling solution was adjusted to 7.4 using either HCl or KOH and the osmolarity was adjusted to 315 mosmol/Kg. Care was taken to ensure that the final concentration of DMSO was < 0.25%. The tip of the electrode was filled with a NE solution whose components are also listed in Table 2. The pH was adjusted to 7.4 and the osmolarity was adjusted to 320 mosmol/Kg. Recordings were obtained using an Axoclamp 2A (Axon Instruments/Molecular devices) bridge amplifier in both the voltage-clamp and the current-clamp mode. The microelectrode resistance was 2–3 MΩ. The electrode junction potential and electrode capacitance were compensated using the amplifier's analogue circuitry. The recording pipette was placed using a Hodgkin-Huxley manual three dimensional microdrive. Drugs were delivered by a multi port gravity feed system (Holt et al., 2003). In the perforated patch experiments, the voltage step protocol involved an initial holding potential of −60 mV (for 7 ms) followed by a step to −120 mV (for 50 ms) followed by a step to 0 mV (for 100 ms) followed by a return to −60 mV (for 150 ms). The protocol is shown as an inset in Figure 4B.

Finally, all the figures were produced using Clampfit V8 (Axon Instruments/Molecular Devices) and/or OriginPro v7.5 (OriginLab, Northhampton, MA). Unless otherwise stated, the data presented are means ± one standard deviation.

Research Highlights

- Pigeon type I and type II hair cells (HCs) have functional cholinergic receptors.
- Activation of these receptors modulates the current and V_z s in pigeon HCs.
- Modulations of the current and V_z s in pigeon type I HCs are previously unreported.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACh	acetylcholine
AChRs	acetylcholine receptors
BK	big conductance - I_{KCa}
BSA	bovine serum albumin
CCh	carbachol
DIC	differential interference contrast
DMPP	1,1-dimethyl-4-phenylpiperazinium iodide
EGTA	ethylene glycol-bis(b-aminoethyl ether)- <i>N,N,N',N'</i> -tetraacetic acid
FBS	fetal bovine serum
HEPES	<i>N</i> -2-hydroxyethylpiperazine- <i>N</i> 9-2-ethanesulfonic acid

HCS	hair cells
I_{KCa}	calcium-activated potassium current
I_{KI} ≡ I_{K,L}	a potassium current found in almost all type I hair cells that activates at low voltages, activates rapidly and slowly inactivates at a V _h of ~-60 mV
I_{IRK1}	inwardly rectifying potassium current - (Kir2.1)
KMeSO₄	potassium methanesulfonate
mAChRs	muscarinic acetylcholine receptors
nAChRs	nicotinic acetylcholine receptors
NE	normal external
PKC	protein kinase C
SCCs	semicircular canals
SK	small conductance - I _{KCa}
TC	tubocurarine chloride hydrate
VHCs	vestibular hair cells
V_h	voltage clamp holding potential
V_z	zero electrode current potential
wk	week

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Figure 1.

Current traces from pigeon type II VHCs elicited during drug treatment and application of the voltage protocol shown in (A) (bottom panel). These traces suggest that depending upon which AChR (mAChR or nAChR) is activated, the current increases or decreases relative to the control. Traces found in (A) and (B) were obtained from the same VHC but those in (C) were obtained from a different VHC. In the traces in (A), 100 μM ACh increased the inward current (●) at -120 mV and the outward current (●) at -60 mV and +40 mV relative to the control trace (□) and represents activation of all cholinergic receptors (mAChRs + nAChRs) present in the cell. However, as shown by the traces in (B) a mixture of 100 μM ACh and 10 μM TC (presumably blocking nAChRs) produces a decrease in the current (●) in the same VHC relative to control (□) at voltages of -60 mV, -120 mV and +40 mV. The remaining current (●) presumably results from mAChR activation. In a different VHC, which produced the traces in (C), the increase in the current induced by 100 μM (●) ACh application is partially recovered (▲) after NE rinse for 15 sec. In (A)-(C) the dash-dotted line indicates the zero current level. In all experiments whose results are presented in this and all subsequent figures, NE was delivered between all drug applications.

Figure 2.

Current traces from a dissociated type II HC during a negative-positive voltage ramp protocol (insert in (A)) and drug delivery. (A) The first application of 100 μ M ACh (●) increases the inward current and outward current compared to the control trace (□). (B) Application of 100 μ M ACh and 10 μ M TC (●) inhibits this response. (C) A second application of 100 μ M ACh to the same HC produces a response (●) similar to the first application (A) but somewhat decreased in amplitude. (D) I/V plots for the 100 μ M ACh-induced current (□) shown in (A) and the 100 μ M ACh + 10 μ M TC inhibited current (●) shown in (B) compared with controls. It is presumed that the inhibited current results from nAChR blockage and mAChR activation. Note that the ACh current as a function of voltage has an N-shape similar to the N-shaped current (I_{KCa}) found in pigeon type II VHCs (Lang and Correia, 1989).

Figure 3.

Membrane potential recordings (obtained in current clamp mode) from dissociated type II HCs. Various AChR agonist and antagonist applications are illustrated (A)-(F). The duration of the drug mixture are denoted by either solid or dotted bars. (A) V_z hyperpolarization during 100 μ M ACh application. (B) V_z hyperpolarization could not be elicited following pretreatment with 100 μ M atropine + 10 μ M TC or 100 μ M ACh + 100 μ M atropine + 10 μ M TC or with post treatment of 100 μ M ACh + 10 μ M TC. These responses suggest that the hyperpolarization was caused by nAChR activation. In contrast, 100 μ M ACh (or 100 μ M CCh) + 10 μ M TC produced hyperpolarization (C, E) or depolarization (D) of the V_z . These results suggest mAChR activation. (E) A large transient hyperpolarization followed by a smaller slower hyperpolarization is shown in response to 100 μ M CCh + 10 μ M TC. (F) The hyperpolarization induced by 2 μ M CCh + 10 μ M TC was blocked by mixture of 100 μ M atropine + 2 μ M CCh + 10 μ M TC. Note that in (A)-(F) we assume that ACh and CCh agonizes both types of cholinergic receptors; 10 μ M TC blocks nAChRs; 100 μ M atropine blocks mAChRs and 100 μ M atropine + 10 μ M TC antagonizes both types of cholinergic receptors. All the effects induced by drug application recovered to near control levels after NE wash. Based on the Nernst equation of our solutions, the E_K in panels 3 (A)-(F) should

be -97 mV at room temperature. It is not known what caused the noisy spikes riding on the V_z traces (E-F) or why some of the spikes exceeded E_K (E-F). However, other publications also show similar noisy deflections during ACh application without a clear causal explanation (Shao and Feldman, 2000; Gusev and Uteshev, 2010; Haj-Dahmane and Andrade, 1996)

Figure 4. Voltage and current clamp recordings from different dissociated Type I HCs that illustrate responses induced by ACh and CCh. (A) An I/V plot using the step protocol described in Suppl. Figure 1A legend. Two μM CCh inhibited the outward current ($\bullet$) 87% (at $+20$ mV) and inward current 35% (at -140 mV). The membrane reversal potential where there was zero net current moved from -68 mV to -55 mV. Following washout of the CCh with NE, the current ($\blacktriangle$) partially recovered to the control level ($\square$). (B) Current responses to a voltage step protocol. The voltage protocol is inset in the figure. The control outward current (denoted by ($\square$) near the trace) was reduced during application of the ACh ($\bullet$). The current was further reduced by the application of $100 \mu\text{M}$ ACh and $10 \mu\text{M}$ TC ($\circ$). Following 150 sec of NE wash, the outward current partially recovered (Δ). (C) Continuous application of $100 \mu\text{M}$ ACh produced an increase in outward current ($\bullet$) after 1.3 s and a further increase in current ($\blacktriangle$) after 2.1 s. (D) An example of depolarization of V_z measured using current clamp. Application of $100 \mu\text{M}$ ACh (dotted bar) depolarized the membrane 26 mV. Subsequent application of $100 \mu\text{M}$ ACh and $10 \mu\text{M}$ TC (solid bar) did not block the effect. The off- response was of greater duration than the on-response during both drug applications. These effects suggest mAChR activation and a metabotropic response. (E) An example of hyperpolarization of V_z by 26 mV following application of $200 \mu\text{M}$ CCh (dotted

bar). Subsequent superfusion of 200 μ M CCh + 10 μ M TC (solid bar) blocked the effect. In (E) the on- and off- responses were roughly of the same duration (off/on ratio of 1.1) and Vz change was blocked by 10 μ M TC. Therefore, it is presumed that nAChRs were activated and an ionotropic response occurred.

Table 1

A summary of responses induced by AChR activation in pigeon type I and type II dissociated vestibular HCs

HCs	Voltage Clamp Mode			Current Clamp Mode	
	Inward current	Current at holding potential	Outward current	Hyperpolarized ΔV_z (mV)	Depolarized ΔV_z (mV)
Type I	nAChR n/o	n/o	↑ Outward Current	Y 26 (n=1)	Y 18 ± 2.8 (n=2)
	mAChR ↑↓ Inward Current	n/o	↓ Outward Current	Y 11 (n=1)	Y 18.8 ± 10.7 (n=4)
Type II	nAChR ↑ Inward Current	↑ Outward Current	↑ Outward Current	Y 10.6 ± 3.0 (n=6)	N
	mAChR ↓ Inward Current	n/o	↓ Outward Current	Y 9.8 ± 4.0 (n=9)	Y 18.3 ± 8.2 (n=6)

* Capital letter Y (N) indicates the presence (absence) of hyperpolarized or depolarized hair cell; mean ΔV_z ± standard deviation (number of observations) is given; n/o indicates change not observed.

Table 2

Concentration of solutions (mM)

	Ruptured Patch Camp		Perforated Patch Clamp	
	External	Internal	Tip	Backfill
NaCl	145	-	-	-
KCl	3	150	140	45
CaCl ₂	2	-	1	1
MgSO ₄	-	-	-	-
MgCl ₂	1	2	2	2
HEPES	15	10	10	10
D-Glucose	10	-	-	3
Na ₂ ATP	-	3	-	-
EGTA	-	-	11	-
Vitamin C	0.283	-	-	-
Na Pyruvate	2	-	-	-
KMeSO ₄	-	-	-	95