Muscarinic ACh Receptor Activation Causes Transmitter Release From Isolated Frog Vestibular Hair Cells

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INTRODUCTION

ACh is the main transmitter at the vestibular efferent-hair cell synapse (reviewed in Guth et al. 1998a). It is released from the efferent nerve terminals innervating type II vestibular hair cells, afferent calyces of type I vestibular hair cells and bouton afferents innervating type-II hair cells. Hair cells isolated from mammalian cochlea (i.e., outer hair cells) (Erostegui et al. 1994; Housley and Ashmore 1991), chick cochlea (Fuchs and Murrow 1992), toadfish saccule (Steinacker and Rojas 1988), frog saccule (Sugai et al. 1992), and frog semicircular canal (Holt et al. 2001, 2003; Housley et al. 1990) all respond electrically to the application of ACh, supporting the presence of an ACh receptor on hair cells, but most of these responses are likely due to the α9/α10 nicotinic receptor (Elgoyhen et al. 2001).

The data obtained from electrophysiological recordings of the afferent firing rate suggest that vestibular semicircular canal hair cells may possess at least three distinguishable ACh receptors. Activation of these ACh receptors on hair cells leads to an inhibition and/or facilitation of afferent firing depending on which ACh receptors are activated. Two of these ACh receptors belong to the nicotinic class of ACh receptors (nAChRs) (Holt et al. 2003).

Evidence does exist for a third hair cell ACh receptor, which is probably muscarinic. The evidence in favor of the existence of this receptor in the vestibular periphery comes from a variety of sources (Drescher et al. 1999; Guth and Norris 1996; Guth et al. 1998b) including multiunit recordings from the frog ampulla nerve (Guth et al. 1986; Holt et al. 2003; Norris et al. 1988; Perin et al. 1998). In this whole-organ preparation, muscarinic receptor activation results in a relatively slow increase in afferent firing. The evidence, though indirect, suggests that this increase in firing depends on an increased release of hair cell transmitter onto afferent endings. However, unlike the nicotinic receptors mentioned, our attempts to stimulate the muscarinic receptors in isolated hair cells have produced no obvious signs of electrical changes. Therefore direct evidence of the concept of a muscarinic receptor existing and functioning on the vestibular hair cell has been clearly lacking. The experiments described in this paper were designed to determine and demonstrate the existence of a functional muscarinic receptor on vestibular hair cells. To produce such evidence, we have attempted to demonstrate that in keeping with the facilitation of afferent firing when muscarinic receptors are activated, isolated SCC hair cells can be made to release their transmitter, thought to be glutamate or a glutamate-like substance (Guth et al. 1998a). To detect the muscarinic receptor-mediated transmitter release from hair cells, these cells were apposed to retinal horizontal cells, which do not express ACh receptors but do express glutamate receptors (Hutchins 1987; Massey and Miller 1987; Massey and Redburn 1987; O’Dell and Christensen 1989; Thoreson and Witkovsky 1999; Whiting et al. 1991). These horizontal cells can therefore act as glutamate detectors. In this study, currents were recorded from voltage-clamped horizontal cells after carbachol application to the hair cells.
M E T H O D S
Electrophysiological recordings of multunit afferent firing from isolated SCC of the leopard frog, Rana pipiens

Electrophysiological recordings were made using the isolated labyrinth of the leopard frog Rana pipiens (Guth et al. 1986). Spontaneous firings of multiple afferent units from the SCC ampullary nerve were recorded as described previously (Holt et al. 2003). Frogs were chilled, double-pithed, and decapitated in accordance with Institutional Animal Care and Use Committee (animal-use committee) standards. The superior portion of the head was sectioned sagittally and placed into artificial perilymph [AP; containing (in mM) 101 NaCl, 2.5 KCl, 5 NaHCO₃, 0.5 NaH₂PO₄, 2.5 Na₂HPO₄, 1.8 CaCl₂, 0.8 MgCl₂, and 5 glucose, pH adjusted to 7.2]. The semicircular canal (SCC) nerve branch was isolated and pulled into a suction electrode filled with AP solution. The whole nerve discharge was amplified 100-fold and filtered (low 1/2: 30 Hz; high 1/2: 10 kHz) through a P-15 amplifier (Grass Instruments, Quincy, MA) and fed into an adjustable window discriminator (Frederick Haer, Brunswick, ME) the output of which was fed on-line into a frequency analyzer set to a 50-Hz bin size to obtain the final frequency-of-firing trace used for off-line analysis. Both fast and slow response amplitudes were temporally resolved and measured using pClamp6 Clampfit software. Fast response amplitudes were measured as the time required to reach the peak fast response from baseline. The fast recovery response was calculated by extrapolating the recovery response to baseline. Slow activation response was calculated as the time it took to reach the slow plateau response from baseline, whereas recovery of the slow response was calculated as the time it took the slow response to reach baseline once it had reached a maximal plateau response.

The labyrinth was placed in a 25-ml bath and continuously perfused with AP at a flow rate of 3–5 ml/min. Drugs were applied either by bath substitution or by close injection (30 s at 50 µl/min) through a multibarrel perfusion pipette the tip of which was placed ~1 mm from the neuroepithelium. The perfusion pipette was linked to a mechanical syringe pump. AP solution was injected prior to each drug application to control for a possible mechanical effect of the injection.

Frog hair cell isolation procedure—initial preparation

The procedure whereby frog SCC hair cells were isolated was described previously by Holt et al. (2001). Briefly, removing the ventral portions of the otic capsule exposed the inner ear. The whole labyrinth was then removed and placed into a dissociation medium containing (in mM) 105 NaCl, 2.5 KCl, 2 MgCl₂, 0.1 CaCl₂, 3.4 NaHCO₃, 0.5 NaH₂PO₄, 2.5 Na₂HPO₄, 1 ascorbate, 4 glucose, and 5 pyruvate. The SCC was subsequently transferred to a dish with Hanks balanced salt solution nominally devoid of calcium and magnesium containing trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA), for 30 min. After trypsinization, the SCC was then transferred into dissociation solution containing 10% fetal bovine serum for a few seconds, and finally into dissociation solution containing 500 µg/ml bovine serum albumin (BSA) for 5–10 min.

Catfish horizontal cell isolation procedure—initial preparation

Isolated catfish horizontal cells were prepared according to the procedure outlined in Linn and Gafka (1999). Dark-adapted channel catfish (Ictalurus punctatus) were anesthetized using tricaine methanesulfonate (100 mg/ml). Both eyes were removed under dim red light. The cornea, lens, and overlying tissue were subsequently excised from each eye and the remaining eyeballs were placed in low-calcium catfish saline containing (in mM) 126 NaCl, 4 KCl, 0.3 CaCl₂, 15 glucose, 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), and hyaluronidase (1 mg/ml), which acts to digest the vitreous fluid covering the retina. After 4 min, the retina was manually peeled off the eyeball and placed in trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA) solution for 4 min. After trypsin treatment, the retina was rinsed well in low-calcium saline and cut into 8–10 pieces. Retinal pieces were stored in normal catfish saline containing 3 mM CaCl₂ and 0.1 mg/ml bovine serum albumin and refrigerated at 5°C until used. Consistent recordings were obtained from these retinal pieces for 24 h (O’Dell and Christensen 1989). Each retina was then further dissociated to yield isolated cone horizontal cells (Linn and Gafka 1999).

Two-cell preparation (hair cell/horizontal cell)

A piece of catfish retina was placed in a petri dish, and the horizontal cells were gently dissociated mechanically in external solution containing (in mM) 101 NaCl, 2.5 KCl, 1.8 CaCl₂, 10 HEPES, 1 ascorbate, 3.6 glucose, 4.5 pyruvate, and 0.01 d-tubocurarine, 0.1 t-glutamate. Glutamate was included to provide for replenishment of glutamate by the cells. Mechanical dissociation of the horizontal cells involved passing the tissue repeatedly through progressively smaller (inside diameter) fire-polished Pasteur pipettes until the retina was completely dissociated. For isolation of hair cells, the cristae of the SCC were removed from the BSA-containing dissociation solution and held in place with forceps on the glass bottom of a 150 µl recording chamber containing external solution and horizontal cells. Under the dissecting microscope, a thin glass wisp was used to loosen the hair cells by a series of gentle mechanical agitations and fluid streaming. Thus both cell types were isolated in the same chamber and as a result the smaller hair cells often came to rest on the surface of the large, flat horizontal cells. Figure 1 depicts a typical example of this two-cell preparation. Large, flat horizontal cells served as detectors by responding electrically when transmitter (i.e., glutamate) was released from the hair cell or when exogenous glutamate was applied.

FIG. 1. The 2-cell apposition preparation. Retinal horizontal cells of the catfish are isolated and placed in the recording chamber. The hair cells are then isolated from the frog semicircular canal (SCC) by gently whisking them over the already isolated horizontal cells. Phase contrast image shows both horizontal cell and hair cell. The arrow indicates the hair cell lying over the horizontal cell surface.
Patch-clamp recordings from isolated cone horizontal cells

The recording chamber containing both the isolated horizontal and hair cells was placed onto the movable stage of a Nikon Diaphot TMD inverted microscope (Nikon, Garden City, NY). The dish was then perfused with external solution at a rate of 1 ml/min for the remainder of the experiments.

The perforated patch-clamp method was used for recordings. The antibiotic, amphotericin B (Sigma, St. Louis, MO), was dissolved in 60 μl of dimethyl sulfoxide (DMSO; Sigma) and homogeneously suspended in 5 ml of internal solution (in mM: 75 KCl, 2 MgCl₂, 30 K₂SO₄, 6 glucose, and 10 HEPES). The final concentration of amphotericin B in the internal solution was 0.6 mg/ml. Borosilicate glass pipettes were pulled from 1.5 mm OD × 0.75 mm ID capillary tubing (Longreach Scientific Resources, Orr's Island, ME) with a Flaming/Brown micropipette puller (model P-97; Sutter Instruments, Novato, CA) and heat-polished (Narashige Scientific Instrument Lab, Tokyo, Japan). Electrodes with resistances measuring between 5 and 10 MΩ were used in this study. Compensation of the tip potential between the pipette’s internal solution and the bath was accomplished before seal formation. The junction potential, based on the constituents of our solutions, was calculated to be approximately −7 mV using the junction potential calculator included with Clampex 6 (Axon Instruments, Foster City, CA). The series resistance was 13 ± 5 (SD) MΩ and partially compensated (70%) using the compensation circuitry of the amplifier. The Axopatch 1B patch-clamp amplifier (Axon Instrument) was used for all voltage-clamp experiments. Horizontal cells were maintained at a holding potential of −60 mV. From this potential, current-voltage relationships were obtained by changing the membrane potential in a ramp wise manner from −100 to +100 mV lasting 350 ms under control conditions and after application of selective agonists and/or antagonists. A paired t-test was used to determine whether currents obtained under control conditions were significantly different from currents obtained following drug treatment. Significance was determined as P < 0.05.

The indicated holding potentials and voltages were not corrected for liquid junction potential. The voltage-clamp data were sampled at 100-μs intervals and low-pass filtered at 5 kHz. Stimuli were generated and data were sampled with a 12-bit D/A and A/D converter (DigiData 1200 series interface, Axon Instruments) and controlled by the data-acquisition software package pClamp6 (Axon Instruments). Voltage-clamp data were stored digitally and later analyzed off-line using the pClamp6 Clampfit (Axon Instruments). Experiments were performed at room temperature (20–22°C). All solutions had an osmolality of 220–230 mosM and pH adjusted to 7.3.

Drugs used

ACh receptor agonists and antagonists were used to distinguish the hair cell’s muscarinic receptor-mediated effects. ACh iodide, carbachol, L-glutamic acid, and AP-5 were also obtained from Sigma. All drug solutions were made up in external solution and applied by a gravity-driven microperfusion pipette at a rate of ~3.6 μl/s. This pipette was placed ~300–500 μm from the cell.

All patch-clamp recordings were done with 10 μM AP-5 in the bath fluid. This concentration of 10 μM AP-5 blocks both known hair cell nicotinic receptors, without affecting muscarinic receptor activity (Elgoyhen et al. 1994; Evans 1996; Guth et al. 1994; Holt et al. 2001, 2003). For inhibition studies using atropine and AP-5, cells were exposed to atropine or AP-5 before and during application of their respective agonists.

RESULTS

Two-cell preparation

Figure 1 illustrates the two-cell preparation in which a SCC hair cell and horizontal cell are apposed. Only the detector cells were monitored by patch-clamping. Isolated horizontal cells range from 25 to 60 μm in length with large dendrites including very fine branches and an attached axon. The overlying isolated SCC hair cell in Fig. 1 (arrow) has a thin elongated trunk measuring 23 μm in length that projects from a base enclosing the nucleus.

Muscarinic receptor-mediated effects on multiunit afferent firing rate recorded from the SCC ampullary nerve

Carbachol (50 μM), a cholinergic agonist, can activate both nicotinic and muscarinic receptors. As shown on Fig. 2A, 50 μM CCh produced a biphasic facilitation of the multiunit afferent firing recorded from the ampullary nerve. A fast phase of the response can be characterized by rapid activation (mean: 151.2 ± 28.3 Hz, n = 5) and recovery with a mean of 33.7 ± 8.5 (SD) s. Unless otherwise stated, reported values are means ± SD. The slow phase of the carbachol response (mean: 88.7 ± 13.1 Hz, n = 5) had relatively slow activation and recovery (mean: 137.4 ± 15 s).

Consistent with previously reported data (Holt et al. 2001, 2003) the fast phase of the carbachol response was blocked completely by 10 μM d-tubocurarine (n = 5), suggesting it is mediated by a nicotinic receptor. The slow response, however, remains intact in the presence of d-tubocurarine and most likely represents the muscarinic receptor-mediated effect (Fig. 2B). The additional application of 1 μM atropine significantly blocked the slow CCh-induced facilitation of the firing rate by a mean of 95 ± 5% (P < 0.01, n = 5; Fig. 2C). The effects of
both antagonists were reversible. After 65 min of washing with AP solution, the muscarinic or slow response returned to control conditions (not shown). All five preparations treated with CCh or ACh in the presence of nicotinic or muscarinic antagonists responded in a similar manner.

To test the hypothesis that muscarinic receptors on frog SCC hair cells can mediate transmitter (glutamate or glutamate-like substance) release, further experiments were performed on a two-cell preparation obtained by pairing isolated frog SCC hair cells with a glutamate-sensitive horizontal cell.

**Horizontal cell sensitivity to glutamate and studies employing the two-cell preparation**

Because glutamate is the presumed transmitter released from the hair cell, this drug was used to test responses of the horizontal cell alone. Solitary horizontal cells were held at a resting membrane potential of −60 mV, and changes in currents in response to glutamate were monitored using a ramp protocol that increased clamping voltage from −100 to +100 mV over 350 ms (Sullivan and Lasater 1992). In the typical example shown in Fig. 3A, when the membrane potential of a voltage-clamped horizontal cell was changed from −100 to +100 mV before glutamate application, the maximal outward current measured was 820 pA and the maximal inward current was −350 pA. After 10 μM glutamate application, the maximal outward current increased to 1,510 pA, whereas the maximal inward current increased to −605 pA. After glutamate washout with control solution, responses returned to control conditions. 80% recovery occurred within 30 s of washout and complete recovery occurred within 2–3 min. For all experiments described in this study, 2–3 min washout periods were used to ensure full recovery. The difference curve obtained by subtracting the control currents from the total currents activated in the presence of glutamate indicates the glutamate-induced current (Fig. 3B). The reversal potential was near 0 mV, which is a characteristic of glutamate ionotropic receptors (Mayer and Westbrook 1984; O’Dell and Christensen 1989).

This response was typical of the results obtained from five of five voltage-clamped catfish cone horizontal cells tested. When the membrane potential of the voltage-clamped cells was changed from −60 to +100 mV, glutamate increased the maximal current significantly (60 ± 32%; P < 0.05) from control conditions. When the membrane potential was changed from −60 to −100 mV, glutamate also significantly increased the maximal inward current by a mean of 67 ± 34% (P < 0.05) compared with control conditions.

As shown previously (O’Dell and Christensen 1989), the horizontal cell’s response to glutamate is mediated by several glutamate receptors including NMDA receptors. Figure 3C represents the difference I–V curve obtained by subtracting the control currents from the total currents activated in the presence of glutamate and in the presence of glutamate and AP-5. As this typical example demonstrates, the exogenous glutamate-evoked response in the horizontal cells was blocked almost completely by the presence of the NMDA receptor antagonist, AP-5 (10 μM), suggesting the primary involvement of NMDA receptors. AP-5 reduced the glutamate response in all voltage-clamped horizontal cells by a mean of 87 ± 7% (n = 5). This represents a significant decrease compared with current traces obtained in the presence of glutamate alone (P < 0.01).

In Fig. 4A, a voltage-clamped horizontal cell’s membrane potential was changed from −100 to +100 mV under control conditions and after application of CCh. The isolated horizontal cells, in the absence of hair cells, never responded to CCh (20–100 μM; Fig. 4A, n = 10) or ACh (50 μM to 1 mM, n = 5; data not shown). In Fig. 4B, the current-voltage relationship from a voltage-clamped horizontal cell was obtained before and after application of glutamate and atropine. Atropine (1 μM) had no significant effect on the glutamate response in the horizontal cell in the absence of hair cells (Fig. 4B, n = 3). Furthermore, atropine, alone, had no significant effect on any horizontal cell in the two-cell preparation (n = 3). In summary, the glutamate response persists in the presence of atropine and is the same with or without atropine.

In contrast, CCh (20 μM) applied to the hair cell in the two-cell preparation always generated responses in the horizontal cell that mimicked responses recorded after glutamate application to the horizontal cell. (Fig. 5A). In this typical example, CCh increased the maximal outward current by 800 pA from control conditions when the membrane potential was changed to +100 mV and increased the maximal inward current by −290 pA from control conditions when the membrane potential was changed to −100 mV. All glutamate-induced responses returned to control conditions after washing with control solution. The difference curve obtained by subtracting the control currents from the total currents recorded from horizontal cell in the two-cell preparation in the presence...
creased the maximal inward current by a mean of 72% when the membrane potential was changed from control conditions. When the membrane potential was held at −60 mV, and I-V curves were generated using the 350-ms ramp protocol (−100 to +100 mV). A: CCh (20 μM) produces no effect on currents recorded from the horizontal cell alone at any voltage. B: the application of 1 μM atropine, a mACh receptor antagonist, had no effect on the horizontal cell response to glutamate (10 μM). Arrows represent control traces. Arrows represent traces obtained in the presence of CCh (A) and in the presence of glutamate and atropine (B).

of CCh is shown in Fig. 5B. The response to CCh was typical of the results obtained from all voltage-clamped catfish cone horizontal cells paired with isolated hair cells (n = 6). When the membrane potential of the voltage-clamped cells was changed from −60 to +100 mV using the ramp stimulus protocol in the presence of CCh, the maximal outward current increased significantly by a mean of 55 ± 22% (P < 0.05) from control conditions. When the membrane potential was changed from −60 to −100 mV, CCh also significantly increased the maximal inward current by a mean of 72 ± 44% (P < 0.05) compared with control conditions. Thus in the two-cell preparation, CCh’s effect on hair cells elicited responses in horizontal cells that were similar to glutamate-produced responses demonstrated in Fig. 3.

Although CCh application to the two-cell preparation elicited significant currents from voltage-clamped horizontal cells in six experiments, the amplitude of the detector (horizontal cell’s) response in the presence of CCh varied from one preparation to another. This variation was most likely due to the amount of transmitter released from hair cells, the number of hair cells per horizontal cell, their arrangement vis-à-vis fluid flow, and glutamate receptor localization. Therefore current-voltage relationship responses due to agonist application were normalized and recorded as percent from controls.

The difference curves demonstrated in Fig. 6 were obtained by subtracting the control currents (currents recorded from the horizontal cell before the application of CCh or atropine) from the total currents recorded in the presence of CCh or in the presence of both CCh and atropine. To obtain this figure, a horizontal cell with overlying hair cells was voltage-clamped at −60 mV and the membrane potential was changed between −100 and +100 mV. Current-voltage responses were obtained under control conditions, after application of CCh, and after application of CCh and atropine. After CCh application, the current elicited at +100 mV significantly increased by 58% (mean increase: 55 ± 22%, P < 0.05). However, if atropine was present, the CCh-induced increase was reduced by 84%. Atropine significantly reduced the CCh-induced increase in the current/voltage response at +100 mV by a mean of 83 ± 12% (P < 0.01) in all voltage-clamped cells containing overlying hair cells (n = 3). At the other end of the voltage range, after CCh application, the current elicited at −100 mV increased by 52% from control conditions (mean increase: 72 ± 44% (P < 0.05). However, in the presence of atropine the CCh-induced increase was reduced by 84%.
atropine, the CCh-induced increase elicited at −100 mV was significantly eliminated in all cases (mean reduction by atropine equalled 98 ± 5% (P < 0.01). Although atropine, at somewhat higher concentrations, is also able to block the α9/α10 nicotinic receptor (Elgoyhen et al. 1994; Verbitsky et al. 2000), 10 μM d-tubocurarine was always present in the extracellular solution to eliminate the possibility of nicotinic ACh receptor activation. Thus mAChRs on hair cells were the most likely targets for the action of CCh.

Suppression of the carbachol effect by a muscarinic receptor antagonist acting on the hair cell and a glutamate receptor antagonist acting on the detector horizontal cell

Further experiments tested whether the glutamate receptor antagonist, AP-5, (a selective NMDA receptor antagonist) was capable of blocking the CCh-induced glutamate effect. To generate Fig. 7, a horizontal cell containing overlying hair cells was voltage-clamped, and the membrane potential was changed under control conditions, after CCh application, and after application of CCh in the presence of AP-5. The current traces shown represent the difference in current obtained by subtracting the control currents from the total currents recorded in the presence of CCh or in the presence of CCh and 10 μM AP-5. As can be seen from the figure, the horizontal cell response to CCh application was significantly suppressed by AP-5 (10 μM). This was typical of results obtained from four other voltage-clamped horizontal cells in the two-cell preparation. AP-5 significantly reduced the CCh-induced response recorded from horizontal cells at all membrane potentials in all four cases. When the membrane potential was changed to +100 mV, AP-5 reduced the CCh-induced response by an average of 85 ± 12% (P < 0.01) and significantly blocked the CCh-induced response when the membrane potential was changed to −100 mV (mean reduction of 95 ± 5% (P < 0.01). In contrast, AP-5 alone produced no significant effect on control currents recorded from any horizontal cells in the two-cell preparation (n = 4), suggesting that isolated hair cells do not spontaneously release transmitter. The activation of muscarinic AChRs, on the other hand, is sufficient to trigger the release of transmitter (glutamate) from the hair cell, which then can activate the AP-5-sensitive NMDA receptors on the detector horizontal cells. Because the horizontal cell response in the two-cell preparation was significantly reduced in the presence of AP-5, NMDA receptors are the most likely candidates mediating the response, although it cannot be ruled out that other glutamate receptors may be involved in this process as the expression of all types of the ionotropic glutamate receptors, AMPA, kainate, and NMDA, has been demonstrated in horizontal cells (O’Dell and Christensen 1989).

DISCUSSION

ACh receptor-mediated facilitation of multiunit afferent firing

It was previously demonstrated (Guth et al. 1986, 1994; Perin et al. 1998) that application of ACh causes a slow facilitation of afferent firing in the frog SCC, which is antagonized by atropine and other muscarinic antagonists but not by the potent α9/α10 nicotinic receptor antagonists, strychnine, or d-tubocurarine (see Fig. 2). CCh is likewise capable of inducing a slow excitation of afferent firing at concentrations selective for muscarinic receptors (Kunitake et al. 2004). The fact that the slow facilitation is blocked by low concentrations of atropine also suggests the involvement of muscarinic receptors. In Xenopus lateral line preparation, Bobbin et al. (1985) saw a facilitation of afferent firing induced by ACh or CCh, which was blocked by atropine (4 μM). Bernard et al. (1985) found that muscarinic agonists caused an increase in frequency of firing recorded from single afferent units in the frog SCC, whereas nicotinic agonists produced both increases and decreases.

At least three distinct responses are observed in the vestibular afferents in response to vestibular efferent stimulation (Bernard et al. 1985; Brichta and Goldberg 2000; Myers et al. 1997; Prigioni et al. 1983). These effects include a rapid inhibition, a rapid facilitation, and a slow facilitation of afferent firing. In the frog, all three of these distinct responses are mimicked by ACh (or other cholinergic agonists) application (Guth et al. 1986, 1994; Holt et al. 2001, 2003). The different effects of efferent stimulation may all be explained by released ACh acting on different receptors. Toward this end, data suggest that the slow afferent facilitation is mediated by a muscarinic receptor. The fast inhibition is most likely mediated by the well-known α9/α10-containing nicotinic receptor (Elgoyhen et al. 1994, 2001). The fast facilitation may be mediated by the recently discovered excitatory nicotinic ACh receptor pharmacologically distinct from α9/α10 (Holt et al. 2003).

Earlier studies in the inner ear were suggestive of the presence of muscarinic receptors on hair cells. Yoshida et al. (1994) using whole cell patch-clamp recordings of isolated saccular hair cells presented evidence for a G-protein-coupled, muscarinic receptor-mediated effect of ACh. These authors proposed the existence of a muscarinic receptor-mediated hyperpolarization. Similarly, Steinacker and Rojas (1988), recording from isolated toadfish saccular hair cells, found that application of ACh or oxotremorine, a muscarinic agonist, caused an increase in open time and opening rate of a K⁺ channel. These effects occurred when the cell-attached mode of recording was used and the drugs were applied in the bath, suggesting the involvement of an intracellular mediator. Note that both of these studies used saccular hair cells the ACh
receptor complement of which may be different from the SCC hair cells used in the present study. At that time, evidence supported the possibility of muscarinic mediation. However, based on later discoveries and cloning of the α9 and α10 nicotinic subunits (Elgoyhen et al. 1994, 2001), it is most likely that the effects, described in those initial studies, were mediated by the hair cell’s nicotinic ACh receptor formed by the α9 and α10 subunits and its colocalization with SK potassium channels (Fuchs and Murrow 1992). Calcium influx through α9/α10 nAChR channels acts as a second messenger to activate the calcium-dependent SK potassium channel (Fuchs and Murrow 1992). Note that the α9/α10 receptor is also sensitive to the classical muscarinic antagonist, atropine (although in higher concentrations), however, weakly activated by the classic nicotinic agonist, nicotine (Elgoyhen et al. 1994; Verbitsky et al. 2000). Oxtremorine, a muscarinic agonist, can also activate the nicotinic α9 homomer expressed in Xenopus oocytes albeit with very low efficacy (Elgoyhen et al. 1994). Wackym et al. (1996) reported on the presence of mRNA of muscarinic receptors M1–M5 in rat vestibular end organs. Finally, Drescher et al. (1999) found binding of the muscarinic ligand, QNB, in sensory epithelial preparations of saccule, utricle, and SCC ampulla of the gerbil. The highest binding was seen in the ampulla nearest to where the efferents invest the hair cells.

One can build a fairly substantial case for the existence of muscarinic receptors in vestibular organs from the evidence cited in the preceding text. However, what was lacking was direct evidence of the presence of functioning muscarinic receptors on vestibular hair cells. Our results clearly demonstrate that the currents evoked in solitary horizontal cells on glutamate application and the currents recorded from detector horizontal cell in the two-cell preparation on CCh application are similar. Thus it is very likely that glutamate or a glutamate-like substance is released from the hair cells on CCh application. The blockade of the glutamate-like response by the NMDA receptor antagonist, AP-5, in the two-cell preparation supports the latter suggestion (Fig. 8). The blockade of this CCh-elicited glutamate-like response by atropine, in concentrations selective for muscarinic receptors, provides strong evidence that it was the activation of muscarinic receptors on CCh application that led to the release of transmitter (glutamate) from hair cells bearing those muscarinic receptors (Fig. 6).

When the two-cell preparation was used, application of 20 μM CCh resulted in horizontal cell current responses that were similar to responses obtained when 10 μM glutamate was applied to solitary horizontal cells. This may suggest that hair cells release a relatively low concentration of glutamate in vivo but more likely is due to the present experimental conditions. Transmitter concentrations released from the hair cells in the two-cell preparation would be greatly diluted before reaching glutamate receptors on horizontal cells. Therefore it is likely that mACHR stimulation, in vivo, releases higher concentrations of glutamate than suggested by this study. In vivo, higher concentrations of glutamate would likely activate AMPA receptors involved in vestibular afferent synaptic transmission. Alternatively, it may be that in vivo activation of mACHRs on hair cells releases glutamate at a concentration that selectively activates NMDA receptors. This is an interesting possibility as a role for NMDA receptors in the peripheral vestibular system has been rather elusive.

Previous studies showed that patch-clamped SCC hair cells do not respond electrically to ACh or CCh in any way other than those mediated by α9/α10 nAChR or the DMPP nAChR (J. C. Holt, unpublished observations). This suggests that activation of the hair cell muscarinic receptors may not produce an effect on hair cell currents. Instead, such activation might elicit calcium release from internal stores, which could be sufficient to trigger transmitter release from the hair cell. Consistently, in our two-cell preparation, the detector horizontal cell generated glutamate-like responses when carbachol was applied to the hair cell. These findings support the hypothesis that CCh may evoke a nonelectrogenic release of the transmitter from the hair cells.

**Proposed nonelectrogenic mechanism of muscarinic receptor-mediated transmitter release from the hair cell**

Several ligands other than ACh acting on metabotropic receptors (e.g., histamine, ATP), while causing increases in afferent firing in whole organ preparations, likewise failed to produce electrical signs of hair cell activation as monitored by patch-clamping methodology (P. Perin, personal communication).

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**FIG. 8.** Schematic illustration of the mechanism of transmitter release from the hair cells in response to muscarinic ACh receptor activation. This figure schematically demonstrates the application of a cholinergic receptor agonist (CCh) on an isolated hair cell to provoke transmitter release, which consequently produces the glutamate-like (Glu) response in the horizontal cell. Note that the nicotinic receptor antagonist, d-tubocurarine, was always present in the extracellular solution eliminating the activation of nicotinic receptors by CCh. The CCh effect was blocked by atropine, a muscarinic antagonist acting on the hair cell, and by AP-5, an NMDA receptor antagonist acting on the horizontal cell, indicating the involvement of muscarinic receptors on hair cells and NMDA receptors on horizontal cells.
tion). The commonality among these ligands is further strengthened when it is realized that all of them activate the same postmetabotropic receptor cascade. On activation of these ligand receptors, phospholipase C acts to release the two separate second messengers, diacylglycerol (DG) and inositol trisphosphate (IP$_3$). Formation of DG activates the enzyme, protein kinase C (PKC), leading to protein regulation, whereas formation of IP$_3$ can lead to increased cytoplasmic calcium due to calcium release from intracellular calcium stores. This commonality among these ligands produced the hypothesis that, in hair cells, metabotropic ligands that cause an increase in afferent firing rates may do so without causing electrical signals such as depolarization and opening of voltage-dependent Ca$^{2+}$ channels. A nonelectrogenic mechanism of transmitter release has been suggested in other systems. For instance, phorbol esters (which activate protein kinase C) enhance transmitter release in hippocampal cell cultures without a change in membrane potential and independent of extracellular Ca$^{2+}$ (Finch and Jackson 1990). Similarly, cAMP (through protein kinase A) (Chen and Regehr 1997; Trudeau et al. 1996) and nitric oxide (Meffert et al. 1996) both cause release of transmitter without involvement of Ca$^{2+}$. The mechanism whereby a nonelectrogenic signal might trigger transmitter release has been offered by Hille et al. (1999). These authors have demonstrated exocytosis without a calcium signal. As with other epithelial cells, hair cells lack action potentials and in them ligands acting on the cascade of G-protein-coupled receptors, second messengers, and protein kinases initiate membrane fusion and secretion. In later publications, this group (Koh et al. 2000) stated that “exocytosis in epithelial cells can be directly stimulated by Ca$^{2+}$, protein kinase A, or protein kinase C.”

Although there is support for suggesting that muscarinally activated transmitter in hair cells may be mediated through a nonvoltage-dependent mechanism as in other cellular systems, care must be taken when analyzing data obtained from enzymatically dissociated isolated cells as enzymatic treatment of cells can alter receptor activity and intracellular enzyme function and lead to false interpretation of results. This is an inherent problem associated with isolated preparations and needs to be addressed for every preparation. Although it cannot be ruled out that mAChR function is altered by trypsin treatment, to determine whether this possibility had validity, whole-organ preparations, which were responding to muscarinic stimulation, were subjected to trypsin at the concentrations used to isolate the hair cells. In three such attempts, the trypsin treatment, if anything, enhanced the response to muscarinic stimulation (J. C. Holt, personal communication). In dissociated catfish horizontal cells, greater evidence exists that receptor function is not significantly altered due to the dissociation process. For instance, in isolated catfish horizontal cells, previous electrophysiological and pharmacological studies have demonstrated that isolated catfish horizontal cells contain functional glutamate receptors that respond in a dose-dependent manner (O’Dell and Christensen 1989) and have identified a series of second-messenger pathways activated after metabotropic glutamate receptor activation and second-messenger pathways involved with modulation of glutamate receptor activity (Davis and Linn 2003; Linn 2000; Linn and Gafka 2001). Furthermore, the electrophysiological and pharmacological characterization of glutamate receptors and second-messenger pathways using isolated catfish cells have been confirmed in a catfish slice preparation, where synaptic connections remain relatively intact (C. Linn, personal communication).

Keeping in mind the culture issues mentioned in the preceding text, the hypothesis that muscarinally mediated transmitter release from hair cells is mediated through a nonelectrogenic mechanism is an idea that has received considerable support from other cellular system. Future studies in the hair cells will address this hypothesis.

**Summary**

The results of this study strongly suggest that the horizontal cell response in the two-cell preparation is induced by transmitter (glutamate) released from the hair cell in response to the activation of muscarinic AChRs on the hair cell. The hair cell transmitter is most likely acting on NMDA receptors on the horizontal cell. That the hair cell transmitter release shown in these studies is in response to muscarinic AChR activation is defended by three facts in this study: it occurs in response to muscarinally selective concentrations of CCh, it is prevented by atropine in muscarinally selective concentrations, and it occurs in the presence of concentrations of d-tubocurarine capable of blocking the known hair cell nicotinic receptors.

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**References**


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