Research Article

Retrograde Facilitation of Efferent Synapses on Cochlear Hair Cells

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ABSTRACT

Cochlear inner hair cells (IHCs) are temporarily innervated by efferent cholinergic fibers prior to the onset of hearing. During low-frequency firing, these efferent synapses have a relatively low probability of transmitter release but facilitate strongly with repetitive stimulation. A retrograde signal from the hair cell to the efferent terminal contributes to this facilitation. When IHCs were treated with the ryanodine receptor agonist, cyclic adenosine phosphoribose (cADPR), release probability of the efferent terminal rose. This effect was quantified by computing the quantum content from a train of 100 suprathreshold stimuli to the efferent fibers. Quantum content was sevenfold higher when IHCs were treated with 100 μ M cADPR (applied in the recording pipette). Since cADPR is membrane impermeant, this result implies that an extracellular messenger travels from the hair cell to the efferent terminal. cADPR is presumed to generate this messenger by increasing cytoplasmic calcium. Consistent with this presumption, voltage-gated calcium flux into the IHC also caused retrograde facilitation of efferent transmission. Retrograde facilitation was observed in IHCs of a vesicular glutamate transporter (VGlut3) null mouse and for wild-type rat hair cells subject to wide-spectrum glutamate receptor blockade, demonstrating that glutamate was unlikely to be the extracellular messenger. Rather, bath application of nitric oxide (NO) donors caused an increase in potassium-evoked efferent transmitter release while the NO scavenger carboxy-PTIO was

able to prevent retrograde facilitation produced by cADPR or IHC depolarization. Thus, hair cell activity can drive retrograde facilitation of efferent input via calcium-dependent production of NO.

Keywords: nitric oxide, hair cell, cochlea, efferent inhibition

INTRODUCTION

Quiescent efferent synapses on cochlear hair cells have a low probability of transmitter release that facilitates markedly with repetitive stimulation (Goutman et al. 2005; Zorilla de San Martin et al. 2010; Ballesterio et al. 2011). Facilitation of transmitter release at the neuromuscular junction was classically attributed to presynaptic calcium accumulation (Katz and Miledi), but a variety of modulatory mechanisms have since been described throughout the nervous system (Nicholls et al. 2012). Of particular interest in the context of synaptic development and stabilization are mechanisms that couple postsynaptic and presynaptic activity. Among these, retrograde facilitation through the calcium-dependent synthesis of the diffusible gas nitric oxide (NO) has been given a prominent role (Garthwaite 2008). The transient efferent innervation of cochlear inner hair cells presents a particularly complex pattern of regulation as these first strengthen after birth, then weaken and disappear at the onset of hearing, 10 to 12 days later (Katz et al. 2004; Roux et al. 2011). Here we report that a rise in hair cell calcium generates a retrograde signal that increases efferent release probability. Since efferent input itself raises hair cell calcium,

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this would provide positive feedback, increasing short-term synaptic strength.

Cholinergic inhibition of hair cells involves the influx of calcium through ionotropic acetylcholine receptors AChRs (Fuchs and Murrow 1992; Oliver et al. 2000) with possible modulation by cytoplasmic calcium stores (Sridhar et al. 1997; Evans et al. 2000). There is an anatomical basis for this hypothesis: hair cells have a distinctive synaptic cistern in close apposition to the efferent synaptic contacts at the base of the cell (Saito 1980). In a previous study, the calcium store release activators ryanodine and cyclic adenosine phosphoribose (cADPR) changed the response to applied ACh and altered the amplitude of spontaneous inhibitory postsynaptic currents (IPSCs) in rat outer hair cells (Lioudyno et al. 2004). Unexplained at the time was a coincident increase in the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs). In the present study, cADPR was infused into rat inner hair cells (IHCs) to promote calcium release through ryanodine-sensitive channels. cADPR increased the amplitude and duration of the SK component of electrically evoked IPSCs, consistent with enhanced release from a postsynaptic calcium store. But in addition, as seen previously, cADPR-treated inner hair cells also had more frequent spontaneous synaptic currents, suggesting an effect on the efferent terminal. Consistent with a presynaptic mechanism, the quantum content of evoked release was significantly greater in cADPR-treated IHCs during electrical stimulation of efferents.

This result implies the existence of a retrograde signal to carry information from the postsynaptic side (IHCs) of the synapse to presynaptic efferent terminals. Retrograde facilitation did not require glutamate release from the hair cell, but rather was prevented by the

NO scavenger carboxy-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide). Activity-dependent retrograde signaling could be a mechanism for first strengthening, then terminating the transient efferent innervation pattern on IHCs, perhaps as a consequence of the developmental downregulation of voltage-gated channels (Beutner and Moser 2001; Marcotti et al. 2003a; Grant and Fuchs 2008) that would lower the overall cytoplasmic calcium concentration.

MATERIALS AND METHODS

Procedures for euthanasia and tissue collection were approved by the Johns Hopkins Institutional Animal Care and Use Committee. Following deep isoflurane anesthesia, rat pups (postnatal days 7–9) were decapitated and the temporal bone removed into cold physiological saline. Apical cochlear turns were microdissected and placed in a recording chamber mounted on an upright microscope (Zeiss Axioskop FS2). The preparation was perfused with saline containing (in millimolar): NaCl (144), KCl (5.8), CaCl_2 (1.3), NaH_2PO_4 (0.7), HEPES (10), and glucose (5.6), pH 7.4 at 2–3 mL/min at room temperature (22–25 °C). Hair cells were visualized through a 40× water immersion objective using green light, DIC optics, and a Newvicon camera (DAGE-MTI). Whole-cell patch clamp recordings from IHCs (343 total, whole-cell capacitance ranged from 7–12 pF) were made under visual control using 4–7 M Ω (initial resistance) pipettes with an internal solution consisting of (millimolar): KCl (135), EGTA (5), HEPES (5), MgCl_2 (3.5), CaCl_2 (0.1), Na_2ATP (2.5), pH 7.3. Series

FIG. 1. Electrical stimulation on efferent axons. **A** The electrical stimulation pipette was positioned ~10–20 mm below the base of the IHC. The aim was to stimulate cholinergic medial olivocochlear efferents that contact IHCs transiently during development. **B** Electrical stimulation (50 μA , 500–800 μs in duration) was at 1 Hz with 100 shocks for each determination of quantum content.

resistance ranged from 6–20 M Ω and was uncompensated for the small signals recorded here.

Efferent axons were stimulated with a bipolar electrode made from 1-mm borosilicate glass (WPI, Sarasota, FL) pulled to a tip diameter of 10–15 μ m and a resistance of 1–2 M Ω . Constant current pulses (~50 μ A, between 500 and 800 μ s in duration) were generated (Digitimer DS3 Digitimer, Welwyn Garden City, UK) and applied to the stimulating electrode through the isolation unit (model DS3). The stimulation pipette was positioned 10–20 μ m below the base of the IHC (Fig. 1). Once reliable activation was achieved, the efferents were stimulated 100 times at 1 Hz to establish release probability, and this protocol was repeated serially to obtain the average quantum content for each condition.

Hair cell membrane currents were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), digitized at 10 kHz, and analyzed off-line using pClamp, Mini Analysis (Synaptosoft, Fort Lee, NJ), and Origin (OriginLab, Northampton, MA) software. Statistical tests were carried out with GraphPad Prism 4 (GraphPad Software, La Jolla, CA). Cyclic adenosine phosphoribose (cADPR, 100 μ M) and ryanodine (Ry, 1 μ M) were added to the intracellular (pipette) solution, and other drugs were delivered through the bath perfusion system. Experimental drugs were obtained from Life Technologies (Carlsbad, CA), Sigma-Aldrich (St. Louis, MO), or Tocris Bioscience (Bristol, UK). Unless stated otherwise, results are presented as mean $\pm$ standard error.

RESULTS

Calcium store activators enhance postsynaptic potassium current

Low-frequency (1 Hz) shocks from a nearby stimulation pipette produced IPSCs in inner hair cells (IHCs) of apical cochlear turns from young (postnatal days 7–9) rats. Under control conditions, IPSCs were evoked infrequently. At -44 mV (E_K , approximately -84 mV) associated potassium currents were outward, with average decay time constants near 65 ms (Fig. 2). Evoked IPSCs in IHCs treated with 100 μ M cADPR (included in the recording pipette's internal solution) were longer lasting (average decay time constant near 90 ms), consistent with the hypothesis that calcium stores modulate synaptic activation of SK channels. In addition, evoked (and spontaneous) IPSCs occurred more frequently, leading to an average evoked response threefold larger than that from control IHCs. It will be shown that this increase in evoked IPSC amplitude reflects an increase in the quantum content (the average number of vesicles released by each action potential) of efferent terminals onto IHCs treated with cADPR.

Activators of calcium store release caused an increase in efferent quantum content

IPSCs recorded at -44 mV are outward-going due to the dominance of the calcium-activated SK conduc-

FIG. 2. Effect of cADPR on ACh-evoked SK current in rat inner hair cells at $V_h = -44$ mV. **A** Representative trace of IPSCs evoked by electrical stimulation of efferent axons. Closed arrows indicate stimulation. Some shocks evoked an IPSC and some failed. Other events occurred spontaneously. **B** Representative trace of IPSCs with 100 μ M cADPR in the recording pipette. Spontaneous and evoked responses were more frequent. **C** Mean evoked IPSC amplitude including failures was 23.47 ± 2.83 pA (SE) in control IHCs (371 events in three IHCs) and was 70.64 ± 5.68 pA for those treated with cADPR (523 events in three IHCs) ($t(2,892) = 6.36$, $p < 0.0001$). **D** Mean evoked IPSC decay time was 64.84 ± 3.1 ms in control (ct) IHCs and 93.03 ± 8.90 ms with cADPR ($t(2,892) = 2.48$, $p = 0.013$).

tance. Compounds that act on cytoplasmic calcium stores, such as cADPR (cyclic adenosine phosphoribose) can alter the amplitude and time course of SK channel gating (Fig. 2) (Lioudyno et al. 2004). To evaluate presynaptic release statistics without these confounding postsynaptic changes, IHCs were voltage-clamped to the equilibrium potential for potassium (-82 mV) so that synaptic currents flowed through the IHC AChRs only (Fig. 3). Under these conditions, it was possible to measure quantal size directly and to calculate quantum content from the relative frequency of evoked synaptic currents (Fig. 4A). Inward currents at -82 mV were smaller and faster than SK-dependent outward currents at -44 mV but could be resolved from the noise. Spontaneous events averaged 10 pA in amplitude while evoked synaptic currents could be fit with a multi-peaked Poisson distribution based on a similar quantal size (Fig. 4A), providing a quantum content of 0.48 for this particular example. Estimates of quantum content were similar whether obtained from the 'direct' method (evoked IPSC/spontaneous IPSC), by the natural log of the fraction of failures or by the reciprocal of the coefficient of variation (Fig. 4B, C). By all these measures, quantum content was consistently larger in IHCs treated with cADPR.

Quantal size (inward current through IHC AChR) was not affected by cADPR (Fig. 5A). However, the average quantum content was sevenfold larger in IHCs treated with cADPR (compared to that in control IHCs (Fig. 5B). Efferent synapses on IHCs treated with the calcium store activator ryanodine (1 μ M) also had larger quantum contents, approximately fourfold greater in ryanodine-treated IHCs compared to untreated cells. These results demonstrate that postsynaptic treatment with compounds that facilitate release from calcium stores increased

FIG. 3. Inward currents through the nAChR of rat inner hair cells evoked at -82 mV (E_K). **A** Representative traces of inward current through the hair cell nAChR evoked by electrical stimulation of efferent axons. SK currents were minimized by recording at the potassium equilibrium potential (-82 mV). **B** Representative traces of IPSCs with 100 μ M cADPR in the recording pipette. Arrows indicate the shock artifact. Note that every stimulus evoked a response, and more spontaneous events were present in the cADPR-treated hair cell.

the probability of transmitter release from the presynaptic efferent terminal, a process of retrograde facilitation. Since cADPR was applied via the intracellular (recording pipette) solution, and is membrane impermeant, this requires that some species of extracellular signaling molecule must travel from the postsynaptic hair cell to the presynaptic efferent terminal to mediate retrograde facilitation. Intracellular cADPR is expected to raise cytoplasmic calcium by release from internal stores and increased the amplitude (Fig. 2) and time course (Fig. 2) of synaptically activated SK currents in agreement with that expectation. Internal stores are not the only source of activity-dependent calcium signaling in hair cells. In addition, voltage-gated calcium channels support action potentials (Kros et al. 1998; Marcotti et al. 2003b) that might mediate activity-dependent maturation of inner hair cells and their afferent connections (Tritsch et al. 2010). To examine the possibility that voltage-gated calcium flux also could cause retrograde facilitation, a protocol was designed that interleaved depolarizing steps to open voltage-gated calcium channels, with electrical stimulation of efferent transmitter release.

Voltage-gated calcium influx caused retrograde facilitation

Efferent shocks at 1 Hz were interspersed with depolarizing steps to -20 mV, near the peak of the current-voltage relation for voltage-gated calcium influx under these experimental conditions (Grant and Fuchs 2008) for 750 ms, 150 ms prior to the next efferent shock (Fig. 6A). After several repetitions of the efferent shock train to establish baseline quantum content, efferent shocks with intervening steps from the holding potential of -82 to -20 mV were begun, producing a marked increase in quantum content (Fig. 6B). In three IHCs, this depolarization protocol produced an average threefold increase in efferent quantum content (Fig. 6C). While not as powerful an intervention as cADPR treatment, the ability to manipulate presynaptic facilitation using postsynaptic voltage commands proved useful in experiments to identify the retrograde signaling mechanism, as will be described.

Glutamate release was not required for retrograde facilitation

Inner hair cells release glutamate to excite postsynaptic afferent neurons (Ruel et al. 1999; Glowatzki and Fuchs 2000). Thus, one possibility is that calcium-dependent retrograde facilitation as described here could be mediated by glutamate released from the IHC. This glutamate might act directly on efferent terminals or through actions on neighboring cell types. This possibility was tested by recording from

FIG. 4. Quantal analysis of cADPR effect in rat inner hair cells. **A** Amplitude distribution of electrically evoked inward currents (eIPSCs) through the hair cell nAChR from one control IHC (bin = 3 pA). Solid red line is the Poisson fit. The dashed blue lines represent the normal distributions of release events including one, two, or three vesicles. Calculated value for quantum content, m , was 0.48 ($n = 252$ evoked IPSCs). **Inset:** amplitude distribution for spontaneous events (sIPSCs) in the same IHC. Solid red line represents Gaussian fit, mean and SE were 10.33 ± 0.33 pA (SE, $n = 154$), corresponding to the quantal size for the evoked histogram. Quantum content was derived from the direct method (evoked/spontaneous event amplitude), by the fraction of failures (**B**) or by the coefficient of variation (**C**). By any of these measures, quantum content was consistently larger in cells treated with cADPR.

IHCs of the VGlut3-null mouse. VGlut-3 is the vesicular glutamate transporter employed by inner hair cells (Seal et al. 2008). Although possessing relatively normal cochlear morphology, this animal is profoundly deaf because IHCs, without a vesicular transporter, fail to release glutamate. Inner hair cell physiology appears otherwise normal, with voltage-gated calcium currents and cholinergic IPSCs like those of wild-type littermates. When IHCs of the VGlut3-null mouse were treated with cADPR, a nearly fivefold increase in quantum content was observed during the efferent stimulation protocol (Fig. 7A). Thus, vesicular glutamate release from mouse IHCs was not required for retrograde facilitation produced by treatment with the calcium store activator cADPR.

To test the possibility that nonvesicular release of glutamate mediates retrograde facilitation, efferent transmission onto IHCs of the rat cochlea treated with cADPR was examined in the presence of glutamate receptor (GluR) antagonists. IHCs were exposed either to a cocktail of metabotropic (mGluR) and ionotropic GluR antagonists ((RS)-alpha-methyl-4-carboxyphenylglycine - MCPG (500 μ M) to block group I/group II metabotropic GluR, (2R)-amino-5-phosphonovaleric acid - AP5 (50 μ M) to block NMDA-type GluRs and 6-cyano-7-nitroquinoxaline-2,3-dione - CNQX (50 μ M) to block AMPA-type GluRs) or to a specific group II mGluR antagonist (LY34149, 200 μ M). In all cases, the efferent quantum content was significantly larger in cADPR-treated cells than in

FIG. 5. Quantal size and quantum content (from fraction of failures) in control rat IHCs (gray) and IHCs treated with cADPR (black). **A** There was no difference in quantal size from four control cells (8.72 ± 0.57 pA, SE) compared to five cells with cADPR (9.57 ± 0.74 pA ($t(2,7) = 0.87$, $p = 0.41$)). **B** Quantum content, m was more than fourfold larger in

cells treated with calcium store release activators ($m = 0.22 \pm 0.02$ for 17 control cells, 2.09 ± 0.33 for eight cells with 100 μ M cADPR ($t(2,23) = 8.32$, $p < 0.0001$), 1.41 ± 0.19 for five cells with 1 μ M Ry, $t(2,20) = 14.01$, $p < 0.0001$ compared to control).

FIG. 6. Depolarization of rat IHCs produced retrograde facilitation. **A** The control protocol included 1 Hz efferent shock trains, recording at -82 mV. In the experimental protocol, each efferent shock was preceded by a 750-ms depolarization of the IHC to -20 mV (ending 150 ms prior to the shock). **B** Diary plot of efferent quantum content (from fraction of failures). After 40 min of repeated 1 Hz of shock trains (every 5 min) to establish

baseline quantum content, intervening depolarizing steps were included, producing a marked increase in quantum content. **C** For 18 control measurements and ten with interleaved depolarization in three IHCs, this manipulation produced a significant increase in quantum content ($m = 0.38 \pm 0.09$ control, $m = 1.13 \pm 0.02$ after depolarization, ($t(2,26) = 6.78$, $p < 0.001$).

matched controls also treated with antagonists (m for control cells, 0.69 ± 0.06 and 3.01 ± 0.42 for cADPR-treated cells, ($t(2,6) = 4.76$, $p < 0.003$). Thus blockade of a spectrum of metabotropic and ionotropic GluRs failed to prevent retrograde facilitation in rat inner hair cells.

Nitric oxide donors increase efferent transmitter release from rat inner hair cells

Since glutamate release was eliminated as the mechanism for retrograde facilitation, other candidates were considered. NO is known to be a potent modulator of synaptic transmission and, in particular, can mediate retrograde facilitation of transmitter release in the central nervous system (Garthwaite

2008). Neuronal nitric oxide synthase is activated by calcium-calmodulin and is reported to be present in the cochlea (Fessenden et al. 1994), so could be a candidate for the calcium-dependent retrograde facilitation seen in these experiments. As a test for its possible involvement, the NO donor SIN-1 (3-morpholiniosydnonimine, 100–250 μ M) was applied in the bath solution while recording from young (P7-9) rat inner hair cells. Since the resting rate of spontaneous release from efferent endings is low, ‘high-potassium’ (40 mM) saline was used first to depolarize efferent endings and increase transmitter release. Several minutes after ongoing exposure to high potassium, the addition of NO donor SIN-1 caused a marked increase in the amplitude and frequency of potassium evoked synaptic

FIG. 7. Enhancement of efferent transmission by cADPR in IHCs of the VGlut3 KO mouse. **A** cADPR increased efferent quantum content, m (from fraction of failures) onto IHCs of VGlut3 KO mice that fail to release vesicular glutamate ($m = 0.68 \pm 0.20$ from four control cells and $3.29 \pm$

0.51 for three cells treated with cADPR, ($t(2,5) = 4.43$, $p < 0.007$). **B** Quantal size was not significantly different with cADPR treatment in the VGlut3 KO OHCs (9.24 ± 0.33 pA and 11.80 ± 0.95 pA ($t(2,5) = 1.77$, $p = 0.09$).

events (Fig. 8). Increased synaptic activity was observed in five of five inner hair cells exposed to 40 mM potassium saline and 100 or 250 μ M SIN-1. Event frequency increased threefold on average (range, 1.4 to 6.2-fold). SIN-1 effects on event amplitude were more variable, with a trend among five inner hair cells to larger events but on average not statistically significant. Of note, this enhancement of event frequency by NO donor was only observed when efferent release onto inner hair cells was accelerated by 40 mM potassium saline. In 5.8 or 20 mM potassium saline, initial rates of release were lower, and exposure to 100–250 μ M NO donors (SIN-1 or SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine) had no significant effect.

Similar experiments were performed on apical turn outer hair cells from older (P12–14) rat cochleas. As observed previously (Wersinger et al. 2010; Ballesteros et al. 2011), efferent synaptic activity was found less commonly in apical outer hair cells and occurred at low rates even in high-potassium (40 mM) saline. Perhaps for this reason, NO donors (SIN-1 or SNAP at 250 μ M) had no effect on potassium-evoked (40, 25, or 20 mM) release in six outer hair cells (four in 40 mM potassium and 250 μ M SNAP, one in 20 mM potassium and 250 μ M SIN-1, one in 25 mM potassium and 250 μ M SIN-1). More extensive studies using evoked release to determine quantum content may be required to examine retrograde facilitation onto outer hair cells in middle or basal cochlear turns where efferent innervation density is higher.

Nitric oxide was required for retrograde facilitation

The effect of NO donors suggested that efferent transmitter release could be modified through the action of NO. However, addition of an NO donor to the bath does not allow one to specify the cellular origin of the NO, and potassium-evoked release does

not readily translate into changes in quantum content. The more acute question is whether activity in the postsynaptic hair cell affects the presynaptic efferent terminal by production of NO. To address this issue, the extracellular scavenger of NO, carboxy-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide) was used to prevent NO-mediated signaling and so was added to the bath around rat IHCs treated with cADPR. In the presence of carboxy-PTIO, the probability of efferent transmitter release onto cADPR-treated IHCs was low but rose upon removal of the scavenger (Fig. 9). In longer lasting recordings, carboxy-PTIO could be applied repeatedly, each time accompanied by a drop in quantum content (Fig. 9A, B). In an IHC with initially high efferent quantum content, application of carboxy-PTIO reduced transmitter release, which rose again after removal of carboxy-PTIO (Fig. 9C).

The NO scavenger carboxy-PTIO also was able to prevent retrograde facilitation produced by IHC depolarization. IHCs were subjected to the efferent stimulation protocol combined with intervening depolarizing steps that were shown to increase efferent quantum content. When conducted in the presence of carboxy-PTIO, the efferent quantum content remained low until the NO scavenger was removed from the bathing solution (Fig. 10). Retrograde facilitation produced by hair cell depolarization was less potent than that due to intracellular cADPR and came on slowly in some cases. Nonetheless, quantum content rose reliably with the depolarization protocol after removal of the scavenger.

DISCUSSION

The present study was undertaken to examine further the role of calcium store activators on efferent inhibition of cochlear hair cells. Earlier work had

FIG. 8. NO donors increased spontaneous transmitter release from efferent terminals onto young rat inner hair cells. Synaptic currents were recorded in IHCs during potassium (40 mM) depolarization and exposure to 250 μ M SIN-1 (shaded region of each diary plot). **A** Several minutes after donor exposure began, spontaneous synaptic

currents became larger. Event amplitude rose from 11.9 ± 0.49 pA to 27.6 ± 0.32 pA ($t(2, 1,597) = 11.65$, $p < 0.0001$). **B** Instantaneous frequency rose from 1.53 ± 0.22 Hz to 7.68 ± 0.22 Hz ($t(2, 1,597) = 6.76$, $p < 0.0001$).

FIG. 9. NO scavenger carboxy-PTIO prevents or reverses retrograde facilitation in rat IHCs treated with cADPR. **A** Diary plot of quantum content (from fraction of failures) determined from repeated 1-Hz shock trains to the efferent axons. Carboxy-PTIO added during the time indicated by gray-shaded regions. Upon removal of carboxy-PTIO from the bath, efferent quantum rose. Quantum content fell again with the return of carboxy-PTIO. **B** In another IHC, repeated presentations of carboxy-PTIO prevented or reversed activity-dependent retrograde facilitation of efferent quantum content. **C** In another IHC with initially

high probability of efferent transmission, carboxy-PTIO reduced efferent quantum content, which recovered after removal of carboxy-PTIO. **D** Efferent quantum content was suppressed by carboxy-PTIO $m=0.42 \pm 0.08$ at 1 Hz ($n=6$) and rose again after removal, $m=1.52 \pm 0.22$ ($t(2,10)=4.69$, $p=0.0009$) ($n=6$). Control data (light gray bars) repeated from Figure 5B for direct comparison. Quantum content for cells treated with cADPR and carboxy-PTIO was significantly larger than that of control cells from Figure 5B ($t(2,21)=3.45$, $p=0.002$).

FIG. 10. NO scavenger carboxy-PTIO prevented depolarization-evoked retrograde facilitation in rat inner hair cells. **A** Diary plot of efferent quantum content (from fraction of failures) derived from repeated efferent shock trains (1 Hz, 100 shocks) that incorporated a 750-ms depolarization to -20 mV 150 ms prior to each shock. Quantum content rose gradually after removal of carboxy-PTIO. **B**, **C**, same protocol repeated in two other IHCs. **D** Carboxy-PTIO (indicated by gray-shaded regions) completely prevented retrograde facilitation normally produced by coupling efferent stimulation with hair cell depolarization ($m=0.20 \pm 0.05$ for six IHCs subjected to the depolarization protocol in the presence of carboxy-PTIO). With removal of carboxy-PTIO, the efferent quantum content increased significantly to 0.94 ± 0.06 ($t(2,10)=5.49$, $p=0.0003$). Quantum content in carboxy-PTIO was not significantly different from that of control data from Figure 5B ($t(2,22)=0.51$, $p=0.62$).

provided evidence that activators of endoplasmic calcium release channels could enhance the amplitude of IPSCs due to ACh release from efferent terminals onto OHCs in the rat cochlea (Lioudyno et al. 2004). Unexplained at the time was an accompanying increase in the frequency of IPSCs evoked by high-potassium saline. The present experiments show that calcium store activators in the postsynaptic hair cell facilitate transmitter release from the presynaptic efferent terminal, in addition to their influence on the postsynaptic calcium signal that activates SK channels. This process of retrograde facilitation does not require glutamate release from the hair cell but can be prevented by extracellular scavengers of NO. The emerging picture is that hair cell activity resulting in elevated cytoplasmic calcium triggers positive feedback to strengthen efferent transmission. Voltage-gated calcium influx also could stimulate retrograde facilitation, but it may be of interest that calcium store release was more potent in doing so. This would accord with the location of the synaptic cistern immediately apposing the efferent synaptic contact while voltage-gated calcium channels are localized to synaptic ribbons serving afferent synaptic function (Meyer et al. 2009). This may suggest that nitric oxide synthase also will be found at the efferent synaptic cistern if immunolabel could be viewed at sufficient resolution. While additional modulatory mechanisms may exist, NO-dependent retrograde facilitation would be local, relatively rapid and mediated by a short-lived chemical signal. The prompt decline of quantum content in the presence of carboxy-PTIO suggests that the downstream effects of NO also degrade rapidly. Finally, the influence of voltage-gated calcium influx suggests that overall levels of cytoplasmic calcium could dictate the efficacy of this feedback mechanism, perhaps by altering the loading of internal calcium stores. These features make this a mechanism for ongoing, activity-dependent modulation of efferent inhibition. NO-mediated enhancement of quantum content may contribute to the activity-dependent facilitation of efferent synapses on inner hair cells (Goutman et al. 2005). Efferent synapses on outer hair cells also facilitate markedly (Ballesterio et al. 2011), although NO donors had no measurable effect on potassium-evoked release in the present study, perhaps because of the far smaller voltage-gated calcium current (Knirsch et al. 2007) and stronger calcium buffering (Hackney et al. 2005) in these cells. Nonetheless, these merits further study since it is known that genetic changes in outer hair cell AChR function lead to altered expression of efferent synaptic proteins (Murthy et al. 2009).

Nitric oxide synthase (NOS) is thought to be expressed in the cochlea (Fessenden et al. 1994). While the lateral wall and vascular epithelia are

predominant sites, supporting cells, afferent neurons, and the sensory hair cells are immunoreactive for NOS and/or can produce NO when stimulated (Shen et al. 2005, 2006). The principal target of NO, guanylyl cyclase is thought to be expressed by cochlear efferents, as is NOS (Riemann and Reuss 1999). Immunogold electron microscopy places NOS in both IHC and OHC and in afferent and efferent nerve fibers (Heinrich et al. 1997). In vestibular hair cells, NO has been reported to reduce voltage-gated calcium currents (Almanza et al. 2007) and voltage-gated potassium currents (Chen and Eatock 2000). In response to ACh, saccular hair cells of the frog produce NO that leads to reduced excitability through combined effects on calcium and potassium channels, possibly a complementary mechanism of efferent inhibition (Lv et al. 2010). Accepting that NOS is present in cochlear hair cells, how might its activation alter release from efferent terminals? NO stimulates guanylyl cyclase to drive formation of cyclic GMP, acting on cGMP-dependent protein kinase G which in turn can have effects on ion channel gating, the vesicular release machinery (Meffert et al. 1996), and calcium regulation (Garthwaite 2008). NO also can alter channel proteins through direct nitrosylation (Bredt and Snyder 1994). One interesting possibility is that NO interacts with voltage-gated channels in the efferent terminal. N, P, Q type calcium channels are thought to support transmitter release, while L-type voltage-gated calcium channels and BK (voltage-gated, calcium-modulated) potassium channels reduce efferent terminal quantum content, presumably by abbreviating the presynaptic action potential (Zorrilla de San Martin et al. 2010). NO could act by altering the gating probability of any of these channels.

At firing rates below a few hertz, efferent quantum content is one or less for synapses on OHCs and young IHCs (Goutman et al. 2005; Ballesterio et al. 2011). Consequently, efferent axons are electrically stimulated at frequencies above 100 Hz to optimize suppression of compound action potentials or distortion product otoacoustic emissions in vivo (Galambos 1956; Gifford and Guinan 1987), and sound-evoked efferent activity can exceed 100 Hz in anaesthetized animals (Robertson and Gummer 1985; Brown et al. 1998). Thus, it is clear that effective inhibition of cochlear hair cells benefits by facilitation of transmitter release from efferent terminals. The retrograde facilitation described here could play a role in this plasticity in outer hair cells. It is less obvious what role facilitation may play at the transient efferent synapses on inner hair cells, although previous observations may provide some clues. Activity-dependent (calcium-dependent) feedback may be required for synaptic stabilization. For example, voltage-gated calcium

channel expression is downregulated in advance of the loss of efferent synapses on IHCs (Marcotti et al. 2003a; Katz et al. 2004; Grant and Fuchs 2008), which would serve to reduce NO-mediated facilitation by reducing overall cytoplasmic calcium, including that of internal stores. What may be critical here is the *change* in calcium influx rather than the absolute level. So, for example, efferent synapses remain intact and functional on IHCs of calcium channel null mice (Brandt et al. 2003), perhaps because there is no drop in calcium influx to disrupt a state of NOS homeostasis and associated presynaptic efficacy. This hypothesis could apply to the permanent efferent synapses on OHCs. Voltage-gated calcium current is smaller in OHCs than in IHCs and has largely completed its developmental downregulation (Knirsch et al. 2007; Beurg et al. 2008) before efferent synapses form (Roux et al. 2011). Thus, efferent synapses on outer hair cells would be secured because they are not subject to reduced retrograde facilitation from lower hair cell calcium, by analogy, to maintained efferent synapses on inner hair cells of calcium channel null mice. Ultimately, these hypotheses must consider interactions with afferent synapses as well. Fewer ribbons and afferents on outer hair cells correspond with greater contact area given over to efferent synapses. Of related interest, efferent synapses return to aged IHCs that have lost some afferent contacts (Lauer et al. 2012). NO-mediated retrograde facilitation is one potential channel for competitive and/or activity-dependent interplay between hair cells and their neuronal partners.

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REFERENCES

- ALMANZA A, NAVARRETE F, VEGA R, SOTO E (2007) Modulation of voltage-gated Ca^{2+} current in vestibular hair cells by nitric oxide. *J Neurophysiol* 97:1188–1195
- BALLESTERO J, DE SAN Z, MARTIN J, GOUTMAN J, ELGOYHEN AB, FUCHS PA, KATZ E (2011) Short-term synaptic plasticity regulates the level of olivocochlear inhibition to auditory hair cells. *J Neurosci* 31:14763–14774
- BEURG M, SAFIEDDINE S, ROUX I, BOULEAU Y, PETIT C, DULON D (2008) Calcium- and otoferlin-dependent exocytosis by immature outer hair cells. *J Neurosci* 28:1798–1803
- BEUTNER D, MOSER T (2001) The presynaptic function of mouse cochlear inner hair cells during development of hearing. *J Neurosci* 21:4593–4599
- BRANDT A, STRIESSNIG J, MOSER T (2003) $\text{CaV}1.3$ channels are essential for development and presynaptic activity of cochlear inner hair cells. *J Neurosci* 23:10832–10840
- BREDT DS, SNYDER SH (1994) Nitric oxide: a physiologic messenger molecule. *Annu Rev Biochem* 63:175–195
- BROWN MC, KUJAWA SG, DUCA ML (1998) Single olivocochlear neurons in the guinea pig. I. Binaural facilitation of responses to high-level noise. *J Neurophysiol* 79:3077–3087
- CHEN JW, EATOCK RA (2000) Major potassium conductance in type I hair cells from rat semicircular canals: characterization and modulation by nitric oxide. *J Neurophysiol* 84:139–151
- EVANS MG, LAGOSTENA L, DARBON P, MAMMANO F (2000) Cholinergic control of membrane conductance and intracellular free Ca^{2+} in outer hair cells of the guinea pig cochlea. *Cell Calcium* 28:195–203
- FESSENDEN JD, COLING DE, SCHACHT J (1994) Detection and characterization of nitric oxide synthase in the mammalian cochlea. *Brain Res* 668:9–15
- FUCHS PA, MURROW BW (1992) Cholinergic inhibition of short (outer) hair cells of the chick's cochlea. *J Neurosci* 12:800–809
- GALAMBOS R (1956) Suppression of auditory nerve activity by stimulation of efferent fibers to the cochlea. *J Neurophysiol* 19:424–437
- GARTHWAITE J (2008) Concepts of neural nitric oxide-mediated transmission. *Eur J Neurosci* 27:2783–2802
- GIFFORD ML, GUINAN JJ JR (1987) Effects of electrical stimulation of medial olivocochlear neurons on ipsilateral and contralateral cochlear responses. *Hear Res* 29:179–194
- GLOWATZKI E, FUCHS PA (2000) Cholinergic synaptic inhibition of inner hair cells in the neonatal mammalian cochlea. *Science* 288:2366–2368
- GOUTMAN JD, FUCHS PA, GLOWATZKI E (2005) Facilitating efferent inhibition of inner hair cells in the cochlea of the neonatal rat. *J Physiol* 566:49–59
- GRANT L, FUCHS P (2008) Calcium, calmodulin-dependent inactivation of calcium channels in inner hair cells of the rat cochlea. *J Neurophysiol* 99(5):2183–2193
- HACKNEY CM, MAHENDRASINGAM S, PENN A, FETTIPLACE R (2005) The concentrations of calcium buffering proteins in mammalian cochlear hair cells. *J Neurosci* 25:7867–7875
- HEINRICH UR, MAURER J, GOSEPATH K, MANN W (1997) Electron microscopic localization of nitric oxide I synthase in the organ of Corti of the guinea pig. *Eur Arch Otorhinolaryngol* 254:396–400
- KATZ E, ELGOYHEN AB, GOMEZ-CASATI ME, KNIPPER M, VETTER DE, FUCHS PA, GLOWATZKI E (2004) Developmental regulation of nicotinic synapses on cochlear inner hair cells. *J Neurosci* 24:7814–7820
- KNIRSCH M, BRANDT N, BRAIG C, KUHN S, HIRT B, MUNKNER S, KNIPPER M, ENGEL J (2007) Persistence of $\text{Ca(v)}1.3$ Ca^{2+} channels in mature outer hair cells supports outer hair cell afferent signaling. *J Neurosci* 27:6442–6451
- KROS CJ, RUPPERSBERG JP, RUSCH A (1998) Expression of a potassium current in inner hair cells during development of hearing in mice. *Nature* 394:281–284
- LAUER AM, FUCHS PA, RYUGO DK, FRANCIS HW (2012) Efferent synapses return to inner hair cells in the aging cochlea. *Neurobiol Aging* 33(12):2892–2902
- LIUDYNO M, HIEL H, KONG JH, KATZ E, WALDMAN E, PARAMESHWARAN-IYER S, GLOWATZKI E, FUCHS PA (2004) A “synaptoplasmic cistern” mediates rapid inhibition of cochlear hair cells. *J Neurosci* 24:11160–11164
- LV P, RODRIGUEZ-CONTRERAS A, KIM HJ, ZHU J, WEI D, CHOONG-RYOUL S, EASTWOOD E, MU K, LEVIC S, SONG H, YEVGENIY PY, SMITH PJ, YAMOA EN (2010) Release and elementary mechanisms of nitric oxide in hair cells. *J Neurophysiol* 103:2494–2505

- MARCOTTI W, JOHNSON SL, RUSCH A, KROS CJ (2003A) Sodium and calcium currents shape action potentials in immature mouse inner hair cells. *J Physiol* 552:743–761
- MARCOTTI W, JOHNSON SL, HOLLEY MC, KROS CJ (2003B) Developmental changes in the expression of potassium currents of embryonic, neonatal and mature mouse inner hair cells. *J Physiol* 548:383–400
- MEFFERT MK, CALAKOS NC, SCHELLER RH, SCHULMAN H (1996) Nitric oxide modulates synaptic vesicle docking fusion reactions. *Neuron* 16:1229–1236
- MEYER AC, FRANK T, KHMICH D, HOCH G, RIEDEL D, CHAPOCHNIKOV NM, YARIN YM, HARKE B, HELL SW, EGNER A, MOSER T (2009) Tuning of synapse number, structure and function in the cochlea. *Nat Neurosci* 12:444–453
- MURTHY V, TARANDA J, ELGOYHEN AB, VETTER DE (2009) Activity of nAChRs containing $\alpha 9$ subunits modulates synapse stabilization via bidirectional signaling programs. *Dev Neurobiol* 69:931–949
- NICHOLLS J, MARTIN A, FUCHS P, BROWN D, DIAMOND M, WEISBLAT D (2012) From neuron to brain. Sinauer Associates, Inc., Sunderland
- OLIVER D, KLOCKER N, SCHUCK J, BAUKROWITZ T, RUPPERSBERG JP, FAKLER B (2000) Gating of Ca^{2+} -activated K^{+} channels controls fast inhibitory synaptic transmission at auditory outer hair cells. *Neuron* 26:595–601
- RIEMANN R, REUSS S (1999) Nitric oxide synthase in identified olivocochlear projection neurons in rat and guinea pig. *Hear Res* 135:181–189
- ROBERTSON D, GUMMER M (1985) Physiological and morphological characterization of efferent neurones in the guinea pig cochlea. *Hear Res* 20:63–77
- ROUX I, WERSINGER E, MCINTOSH JM, FUCHS PA, GLOWATZKI E (2011) Onset of cholinergic efferent synaptic function in sensory hair cells of the rat cochlea. *J Neurosci* 31:15092–15101
- RUEL J, CHEN C, PUJOL R, BOBBIN RP, PUEL JL (1999) AMPA-preferring glutamate receptors in cochlear physiology of adult guinea-pig. *J Physiol* 518:667–680
- SAITO K (1980) Fine structure of the sensory epithelium of the guinea pig organ of Corti: afferent and efferent synapses of hair cells. *J Ultrastruct Res* 71:222–232
- SEAL RP, AKIL O, YI E, WEBER CM, GRANT L, YOO J, CLAUSE A, KANDLER K, NOEBELS JL, GLOWATZKI E, LUSTIG LR, EDWARDS RH (2008) Sensorineural deafness and seizures in mice lacking vesicular glutamate transporter 3. *Neuron* 57:263–275
- SHEN J, HARADA N, NAKAZAWA H, YAMASHITA T (2005) Involvement of the nitric oxide-cyclic GMP pathway and neuronal nitric oxide synthase in ATP-induced Ca^{2+} signalling in cochlear inner hair cells. *Eur J Neurosci* 21:2912–2922
- SHEN J, HARADA N, NAKAZAWA H, KANEKO T, IZUMIKAWA M, YAMASHITA T (2006) Role of nitric oxide on ATP-induced Ca^{2+} signaling in outer hair cells of the guinea pig cochlea. *Brain Res* 1081:101–112
- SRIDHAR TS, BROWN MC, SEWELL WF (1997) Unique postsynaptic signaling at the hair cell efferent synapse permits calcium to evoke changes on two time scales. *J Neurosci* 17:428–437
- TRITSCH NX, RODRIGUEZ-CONTRERAS A, CRINS TT, WANG HC, BORST JG, BERGLES DE (2010) Calcium action potentials in hair cells pattern auditory neuron activity before hearing onset. *Nat Neurosci* 13:1050–1052
- WERSINGER E, McLEAN WJ, FUCHS PA, PYOTT SJ (2010) BK channels mediate cholinergic inhibition of high frequency cochlear hair cells. *PLoS One* 5:e13836
- ZORRILLA DE SAN MARTIN J, PYOTT S, BALLESTERO J, KATZ E (2010) Ca^{2+} and Ca^{2+} -activated K^{+} channels that support and modulate transmitter release at the olivocochlear efferent-inner hair cell synapse. *J Neurosci* 30:12157–12167