Evidence that protons act as neurotransmitters at vestibular hair cell–calyx afferent synapses

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Present data support the conclusion that protons serve as an important neurotransmitter to convey excitatory stimuli from inner ear type I vestibular hair cells to postsynaptic calyx nerve terminals. Time-resolved pH imaging revealed stimulus-evoked extrusion of protons from hair cells and a subsequent buildup of $[H^+]$ within the confined chalice-shaped synaptic cleft ($\Delta pH \sim 0.2$). Whole-cell voltage-clamp recordings revealed a concomitant nonquantal excitatory postsynaptic current in the calyx terminal that was causally modulated by cleft acidification. The time course of $[H^+]$ buildup limits the speed of this intercellular signaling mechanism, but for tonic signals such as gravity, protonergic transmission offers a significant metabolic advantage over quantal excitatory postsynaptic currents—an advantage that may have driven the proliferation of postsynaptic calyx terminals in the inner ear vestibular organs of contemporary amniotes.

Informal transmission in the nervous system occurs primarily through chemical signaling by small molecules packaged in synaptic vesicles and released quantally (1, 2) and gaseous free radicals that diffuse through 3D tissue volumes, oblivious to membrane boundaries (3–5). These two chemical modes of neurotransmission often act synergistically and in some systems, are augmented by electrical ephaptic interactions (6) and gap junctions (7). Here, we report a third mode of chemical intercellular signaling between inner ear type I hair cells and calyceal afferents: nonquantal protonergic transmission. Previously, proton release from the intestine during defecation was shown in Caenorhabditis elegans to activate a proton-gated ion channel in the adjacent muscle, thus showing that protons are an important transmitter in executing that motor program (8). Present results demonstrate the generality of this finding by documenting protonergic neural transmission in the inner ear.

The inner ear vestibular sensory epithelia of reptiles, birds, and mammals contain two hair cell types, I and II (both presynaptic to primary afferent processes) (9). Chalice-shaped terminals envelop one or more type I hair cells, forming synaptic contacts with the inner leaflet of the calyx terminal. Type II cells innervate bouton terminals and in some cases, the outer leaflets of calyces (10, 11). Synaptic transmission between type II cells and their terminals is chemically mediated and quantal (12, 13). Synaptic transmission between type I cells and their calyces has a quantal glatameric component (13–17) augmented by a nonquantal excitatory postsynaptic current (nqEPSC) (14, 16).

It was hypothesized previously that the ball-and-socket morphology of the hair cell–calyx terminal might lead to the nqEPSC through stimulus-evoked modulation of the $[K^+]$ within the synaptic cleft (18–20). Present results support an alternative protonergic signaling mechanism, in which stimulus-evoked acidification of the cleft leads to modulation of a postsynaptic cationic conductance.

Significance

Recent evidence from the neuromuscular junction in Drosophiia and C. elegans shows that protons are important intercellular signaling molecules operating to modulate presynaptic release or ion channels in adjacent cells. Here, we present evidence that protons also act directly as a nonquantal neurotransmitter in a contemporary amniote to convey excitatory stimuli from type I inner ear vestibular hair cells to their partner postsynaptic calyx nerve terminals. Protonergic neurotransmission works in concert with classical mechanisms and endows this system with a metabolically efficient mechanism to evoke tonic depolarizations of the postsynaptic neuron. Similar intercellular proton signaling mechanisms might be at play in the CNS.

Results

Whole-cell voltage-clamp recordings from calyx nerve terminals in the vestibular lagena revealed slowly developing nqEPSCs and quantal excitatory postsynaptic currents (EPSCs) evoked by deflection of a single type I hair cell bundle ($\sim$60 mV hold). The experimental setup is illustrated in Fig. 1. For fluid jet mechanical stimuli, the bundle moved with a time constant of $\sim$29 ms and reached an $\sim$1-μm displacement within 20 ms of the stimulus onset, and for stiff-probe stimuli, it reached an $\sim$1-μm displacement within 2 ms. Whole-cell capacitance averaged 27.3 pF (SD = 9.05) and series resistance averaged 38.0 MΩ (SD = 8.12). Pipette resistance was nominally 6 MΩ. In a majority of calyces (n = 36; 70%), deflection of a single hair cell bundle evoked a tonic nqEPSC without changing the rate of quantal EPSCs (Fig. 2A). In seven calyces (14%), bundle deflection evoked an increase in the rate of quantal EPSCs without evoking an nqEPSC (Fig. 2B), and in eight calyces (16%), bundle deflection simultaneously evoked an nqEPSC and an increased rate of quantal EPSCs (Fig. 2C). The EPSC rate adaptation time constant averaged 899 ms (115 ms minimum and 2,760 ms maximum; n = 9), excluding one cell that did not exhibit measurable adaptation. nqEPSCs did not adapt. This contrast is most easily seen in Fig. 2C, where the rate of quantal release declined during bundle deflection, whereas the nqEPSC reached a tonic plateau. nqEPSCs, therefore, offer an advantage over quantal EPSCs for conveying tonic or slowly changing signals. Quantal and nonquantal currents could be evoked simultaneously by deflection of a single hair bundle (Fig. 2C). A single hair cell is capable of conveying tonic depolarization through the nqEPSC and phasic information by adapting quantal release. This signaling implies the presence of a mechanism to adapt the rate of presynaptic vesicle release that does not interfere with synaptic transmission | indefatigable


The authors declare no conflict of interest.

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tonic nonquantal neurotransmission. Application of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 50 μM) reversibly blocked EPSCs but did not alter nqEPSC amplitude or kinetics (Fig. 2A) (n = 2), confirming a previous report showing that nqEPSCs were completely independent of glutamate receptor action (14). The origin of the nqEPSCs is the focus of the present report.

The amplitude of the nqEPSC for saturating stimuli was 106.2 pA (SE = 9.04 pA; −60 mV hold; n = 41). In 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffered media, the average rise time was $\tau_{\text{on}} = 436$ ms (SE = 40 ms), and decay time was $\tau_{\text{off}} = 318$ ms (SE = 44 ms). nqEPSC time constants were not limited by the 29-ms time constant of the fluid jet-driven hair bundle displacements. Fig. 3A and B report the voltage sensitivity of the nqEPSC. The reversal potential shifted from +44 to +15 mV in these cells when N-methyl-d-glucamine (NMDG+) replaced media Na$^+$ and on average, shifted from +56 to −6 mV (n = 5; average whole-cell C = 24.8 pF, R = 52.8 MΩ, electrode $R_E = 5.6$ MΩ). Reducing media [Ca$^{2+}$] from 2.8 to 0.1 mM had no effect on the nqEPSCs. The time constant was not sensitive to the holding potential, with the exception of a singularity that occurred near the reversal potential, which would be expected if the channel is not perfectly selective for a single ion. The fact that the current reversed monotonically (i.e., the rising and falling phases reversed at the same potentials as the plateau) shows that the nqEPSC was not caused by a buildup of the charge carrier(s) in the cleft. To confirm dependence on a change in membrane conductance, we recorded whole-cell resistance using a 256-Hz interrogating sine wave superimposed on the voltage-clamp command (Fig. 3C) (n = 3). Kinetics and magnitude of the conductance change were sufficient to explain the nqEPSC without any change in the electrochemical
potential during the stimulus. Data strongly suggest a stimulus-evoked chemical neurotransmitter released by hair cells into the cleft, leading to gating of a cation conductance.

To estimate charge carrier concentrations in the cleft, we used the known patch pipette solution and zero current potentials measured in N- (Dithioacarbonyloxy)-N-methyl-D-glucamine sodium salt monohydrate (NMDG) and control media. Because we were unable to alter the nqEPSC with low extracellular \( [Ca^{2+}] \) (Fig. 3A), we assumed the current was carried primarily by \( Na^+ \) and \( K^+ \). Numerical optimization of Goldman–Hodgkin–Katz parameters (SI Text) estimated a relative permeability of \( P_{Na,K} = 5.2 \), \( [K^+]_{\text{CLEFT}} = 84 \text{ mM} \), and \( [Na^+]_{\text{CLEFT}} = 41 \text{ mM} \) in normal media as well as \( [K^+]_{\text{NMDG}} = 115 \text{ mM} \) and \( [Na^+]_{\text{NMDG}} = 10 \text{ mM} \) in the presence of NMDG. These values reproduce the voltage sensitivity reported in Fig. 3 and indicate that the nqEPSC is driven, in part, by unusually high extracellular \( [K^+] \) and low \( [Na^+] \) in the cleft.

The relatively slow onset of the nqEPSC suggested that a stimulus-evoked signaling molecule might build up in the cleft and modulate the nqEPSC. Because increased metabolic activity leads to proton extrusion from hair cells, we examined changes in cleft pH as a candidate signal during hair bundle deflections. Fig. 4 (n = 6) illustrates the magnitude (Fig. 4A) and kinetics (Fig. 4B) of stimulus-evoked pH changes in the cleft. The hair bundle was imaged under control and stimulated conditions to quantify the stimulus (Fig. 4A, i–iii) (~1 μm). Focusing down ~12 μm below the cuticular plate revealed regions of high ratiometric membrane impermanent carboxylic acid, acetate, succinimyl ester (SNARF, Life Technologies) concentration in the extracellular space around the hair cell. The fluorescent region was much larger than the extracellular synaptic cleft, presumably because of the 3D morphology, dye outside the calyx, and out-of-focus light (>5-μm optical slice). Ratiometric imaging was used to estimate the spatial distribution of pH in the control (Fig. 4A, vii) and stimulated (Fig. 4A, viii) conditions. The difference between the two provided acidification of the cleft. Punctate regions of high acidification (Fig. 4A, ix) were visible, suggesting that proton extrusion from hair cells might be spatially localized. For this specific cell, cleft acidification averaged \( \Delta \text{pH} = -0.19 \) (~0.21 at puncta), and cleft acidification over all tested cells averaged \( \Delta \text{pH} = -0.20 \) (n = 4, peak change at puncta, SD = 0.046). Because the highest \( \Delta \text{pH} \) always occurred around the stimulated hair cell and not around other hair cells enveloped by the same calyx, we concluded that the stimulated hair cell was the source of protons entering the cleft.

Time-resolved confocal microscopy (21) was used to examine the kinetics of cleft acidification (n = 3). Fig. 4B, i provides the spatially averaged \( \Delta F/F \) in the cleft surrounding stimulated hair cell 1 (green squares, Fig. S1). Imaging limitations likely account for the inability to resolve \( \Delta \text{pH} \) puncta using this system and the small modulation in the center of hair cell 2 (black circles). We also analyzed \( \Delta F/F \) in the Fourier domain to find the amplitude (Fig. 4B, ii) and phase (Fig. 4B, iii) (delay to peak \( \Delta F/F \)) as functions of cleft position. The size of each symbol denotes the magnitude of modulation or relative delay for the region (~1 μm) of the image directly under the symbol. Stimulus-evoked cleft acidification was largest in the ring surrounding the stimulated hair cell and declined as the distance from the stimulated cell increased (Movie S1). Peak modulation was delayed ~430 ms in distant regions relative to those adjacent to the stimulated hair cell. Results show injection of protons from the stimulated hair cell into the cleft, effective held at ~60 mV showing the reversible reduction of quantal EPSCs with little change in the nqEPSC in NMDG (blue traces). (C) nqEPSC was caused by a change in conductance.
concentration of bound and unbound buffer in the cleft, and diffusion of protons away from the stimulated hair cell, and cycle-by-cycle clearance of protons.

The onset time constant of cleft acidification averaged 558 ms ($n = 5, SD = 49$) and was well within the range of the nqEPSC onset time constants recorded in the calyx [436 ms in 10 mM Hepes and 697 ms in 20 mM piperazine-$N,N'$-bis(2-ethanesulfonic acid) (PIPES)]. This correlation led us to hypothesize that protons might be the neurotransmitter leading to modulation of nqEPSCs, through direct (Fig. S2) or indirect action on a postsynaptic cation channel. If true, nqEPSC kinetics would be sensitive to pH buffering. To explore pH sensitivity, we modeled the cleft using a single compartment as illustrated in Fig. 5A. We assumed that protons were extruded from hair cells into the cleft at a stimulus-dependent rate $q(i)$ and buffered by a reversible second-order reaction within cleft volume $V$. Cleft pH homeostasis was modeled using a proportional mechanism driving free $[H^+]$ to baseline $[H^+]_0$ with gain $k$. For quasiequilibrium buffering, mass balance yields the model in Fig. 5A, where $[B]_0$ is the total concentration of bound and unbound buffer in the cleft, and $K_d$ is the proton–buffer dissociation constant. Free proton concentration $[H^+]$ predicted by this model was used to drive a model of a ligand-gated ion channel (SI Text) to simulate nqEPSC kinetics (Fig. 5B, i).

Based on this analysis, increasing the strength of the buffer is predicted to slow the nqEPSC kinetics but not change the steady-state amplitude caused by proton homeostasis in the cleft. To test this prediction, we applied a series of extracellular pH buffers, all adjusted to a common pH, and recorded nqEPSC kinetics in control (10 mM Hepes, $n = 37$), test (1 mM Hepes, $n = 8$; 20 mM PIPES, $n = 29$), and then washout control conditions for individual cells. Time constants of the nqEPSC onset ($\tau_{ON}$) and recovery ($\tau_{OFF}$) in the control condition (10 mM Hepes) were used to define a characteristic nqEPSC time constant of each cell $[\tau_c = (\tau_{ON} + \tau_{OFF})/2]$ (Fig. 5B). Results for each cell were normalized by $\tau_c$ to compensate for differences in calyx size (note $V$ in Fig. 5A and morphological diversity Fig. S3). Application of 1 mM Hepes (pK $\sim 7.4$) significantly decreased the time constants, whereas 20 mM PIPES (pK $\sim 6.8$) significantly increased them ($n = 5, P < 0.001$). All changes reversed with washout. Consistent with the data, the model predicted that PIPES should increase the time constant $\sim 1.7$-fold over the control buffer. These results show that nqEPSC is causally linked to free $[H^+]$ in the cleft.

**Discussion**

The vestibular calyx nerve ending is unique in the extent to which it envelops its presynaptic partners. This morphology maximizes the ability of the calyx to capture molecules exiting hair cells. Present results document capture of protons and modulation of a nonquantal postsynaptic cationic current in the vestibular calyx synaptic terminal. The strongest evidence for causal protonergic neurotransmission is the predictable effect of extracellular pH buffers on the kinetics of nqEPSCs (Fig. 5). It has been shown previously that stimulus-evoked acidification modulates quantal synaptic transmission through action on both Ca$^{2+}$-dependent and -independent vesicular release pathways (22). This dependency raises the possibility that stimulus-evoked acidification of the cleft might also be linked to a protonergic feedback signal influencing quantal release and adaptation (23). Results support the general conclusion that protons serve as important intercellular transmitters (8) and synaptic signaling molecules (22, 23).

Because nqEPSCs were often present in the absence of quantal release (Fig. 2), and because puncta of high APH were observed at locations distant from sites of quantal release (Fig. 4), we can rule out synaptic vesicles as the primary source of cleft acidification (24, 25). It is more likely that protons were extruded into the cleft during hair cell depolarization through mechanisms such as Na$^+$ /H$^+$ exchange or electrogenic Na$^+$ /HCO$_3^-$ cotransport. The identity of the postsynaptic channel is unknown, and present results cannot distinguish whether the nqEPSC was directly gated by protons or a second messenger. It is likely that proton-sensitive ion channels are present in vestibular calyx terminals (SI Text, Fig. S2), but the extent to which they may contribute to the nqEPSC is not yet known.

![Fig. 4](image-url)
A Kinetic Model of Cleft pH

\[ \frac{d}{dt} \left( [H^+] \right) = \frac{g}{k_d} \left( k_{d}^{-1} [H^+] - [H^+] \right) \]

\( k_d \) is the proton diffusion coefficient in the cleft under physiological conditions. The parameter is not classical, but includes effects of pH buffers and proton homeostasis. We estimate that 10 mM Hepes retarded the buildup of \([H^+]\) in the cleft and slowed the nqEPSCs at least threefold relative to endogenous conditions (Fig. 5) (asymptote with zero exogenous buffer), giving physiological estimates for the nqEPSC time constants of \( \tau_{ON} \lesssim 145 \text{ ms} \) and \( \tau_{OFF} \lesssim 106 \text{ ms} \). Results also suggest that the effective proton diffusion coefficient in the cleft under physiological conditions is \( \sim 5 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1} \) or higher. This effective diffusion coefficient is very fast (like a shot) relative to proton diffusion in other systems (28) and implies that endogenous proton buffering is weak. Our findings also raise the possibility that the morphology of the cleft enhances long-range proton migration through the interaction between hydrated protons and the tightly juxtaposed membranes (29–31). Results show the importance of both morphology and endogenous pH buffering in determining pH kinetics in the cleft. nqEPSCs recorded in utricular calyces of immature rats (16) are considerably faster than in the adult turtle lageneral calyces examined here. The relatively small volume of the calyx synaptic cleft in young rats clearly would increase the speed of proton buildup relative to the turtle lagena (Fig. 5), accounting for at least some of the interspecies differences in nqEPSC kinetics.

B nqEPSC Time Constant with Buffers

![Diagram showing nqEPSC time constant with buffers](image)

Fig. 5. Kinetics of cleft acidification were altered by pH buffers. (A) Single compartment model of the cleft accounting for stimulus-evoked proton input from the hair cell, reversible pH buffering within the cleft, and pH regulation by endogenous mechanisms. Buffering was assumed to be fast and in quasiequilibrium. (B) Exogenous buffers after kinetics of nqEPSCs. (i) The model predicted that increasing buffer concentration would slow the speed of cleft acidification (because of buffer concentration \( B_b \) and \( K_d \)) with no change in steady-state magnitude caused by endogenous pH regulation. (ii) Consistent with this model, nqEPSC onset (\( \tau_{ON} \)), recovery time constants (\( \tau_{OFF} \)), and average time constants \( [\tau = (\tau_{ON} + \tau_{OFF})/2] \) increased with pH buffer strength (\( n = 4 \)). Time constants were normalized for each cell by \( n = r \) recorded in the same cell using 10 mM Hepes. (iii) Onset and recovery time constants both increased with the strength of the buffer and returned to control values on wash to 10 mM Hepes (\( p < 0.05, n = 4 \), average of 6–29 records/cell, error bars show SD of \( \tau_r/\tau_c \), between cells).

Present results suggest that the nqEPSC is carried by \( Na^+ \) and \( K^+ \) with a relative permeability of \( \sim P_{Na}/P_{K} = 5.2 \). Voltage sensitivity of the nqEPSC indicated that resting \( [K^+] \) was maintained at an unusually high level in the cleft. It is likely that high cleft \( [K^+] \) is facilitated by stimulus-evoked \( K^+ \) currents from hair cells (19, 20), and the low voltage-activating \( K^+ \) current present only in type I hair cells (26). Ionic homeostasis in the cleft is, therefore, critical and likely to be executed in part by the complex spatial array of channel proteins expressed in the calyx (27).

The \([H^+]\) transients evoked in remote regions of the cleft were delayed relative to transients around the stimulated hair cell. The delay suggests an effective proton diffusion coefficient of \( \sim 1.5 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1} \). This parameter is not classical, but includes effects of pH buffers and proton homeostasis. We estimate that 10 mM Hepes retarded the buildup of \([H^+]\) in the cleft and slowed the nqEPSCs at least threefold relative to endogenous conditions (Fig. 5) (asymptote with zero exogenous buffer), giving physiological estimates for the nqEPSC time constants of \( \tau_{ON} \lesssim 145 \text{ ms} \) and \( \tau_{OFF} \lesssim 106 \text{ ms} \). Results also suggest that the effective proton diffusion coefficient in the cleft under physiological conditions is \( \sim 5 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1} \) or higher. This effective diffusion coefficient is very fast (like a shot) relative to proton diffusion in other systems (28) and implies that endogenous proton buffering is weak. Our findings also raise the possibility that the morphology of the cleft enhances long-range proton migration through the interaction between hydrated protons and the tightly juxtaposed membranes (29–31). Results show the importance of both morphology and endogenous pH buffering in determining pH kinetics in the cleft. nqEPSCs recorded in utricular calyces of immature rats (16) are considerably faster than in the adult turtle lageneral calyces examined here. The relatively small volume of the calyx synaptic cleft in young rats clearly would increase the speed of proton buildup relative to the turtle lagena (Fig. 5), accounting for at least some of the interspecies differences in nqEPSC kinetics.

Because protons are a byproduct of glycolytic metabolism and ATP hydrolysis, extrusion of protons by hair cells and capture by the calyx ending would constitute an energetically efficient mechanism for signaling tonic hair cell activation, whereas vesicular release would signal transient activation at a higher energetic cost. This energy efficiency may explain why nqEPSCs and calyx terminals are prolific in modern vestibular hair cell organs responsible for sensing tonic signals such as gravity, but absent from auditory hair cell organs responsible for sensing fast transient signals (12, 32).

Materials and Methods

Procedures complied with the Institutional Animal Care and Use Committee regulations at the Marine Biological Laboratory. Electrophysiological and imaging data were obtained from lagena of adult red-eared slider turtles, Trachemys scripta elegans. The otolithic membrane and otoconia were removed by incubation in type XXIV proteinase (0.06 mg mL$^{-1}$ in 0.1 mM calcium Ringer’s solution; P8038, Sigma-Aldrich). Epithelia were superfused with an oxygenated Ringer’s solution: 125 mM NaCl, 4 mM KCl, 2.2 mM MgCl$_2$, 8.2 mM CaCl$_2$, 8 mM o-glucose, and 1–10 mM Na-Hepes. Alternatively, 20 mM PIPES buffer was substituted for Hepes. NDMDG$^+$ was substituted for Na$^+$ in some experiments. All extracellular solutions were pH-balanced to 7.53. CNQX (C$_9$H$_4$N$_4$O$_4$) was bath-applied (50 μM). Bath perfusion was continuous at 3 mL min$^{-1}$.

Patch electrodes contained 120 mM KCl, 2.8 mM MgCl$_2$, 0.45 mM CaCl$_2$, 5 mM EGTA, 2.5 mM Na$_2$ATP, and 10 mM K-Hepes (pH 7.2) often in a 2–4% by weight solution of Lucifer yellow (Sigma-Aldrich) or 125 mM CsCl, 2.8 mM MgCl$_2$, 5.5 mM EGTA, 0.45 mM CaCl$_2$, 5 mM Hepes, and 2.5 mM Na$_2$ATP (pH 7.5). Experiments were performed using a 63x, 1.0-N.A. objective on a fixed-stage Zeiss Axiophot F31 equipped with a swept field confocal (Prairie Technology). Experimental protocols were computer-controlled (PatchMaster, Heka). An Axon 700b amplifier (Molecular Devices) was used for whole-cell recording. Analog signals were recorded at 16 bits, filtered at 16 kHz (npi electronic GmbH), and sampled at 96 kHz (Agospede AD16x modified for DC coupling; IGOR Pro; WaveMetrics). Whole-cell capacitance was compensated online, and series resistance was compensated offline (S1 Text).

Hair cell bundles were stimulated with a fluid jet attached to a picospritzer (1A, pH 800; WPI) or in a subset of experiments, by a closed-loop displacement-controlled stiff glass probe (P-841.3; Physiwick Instrumente). For fluid jet stimuli, the magnitude of the bundle movement, rise time, and delay relative to the electrical trigger were determined in a subset of experiments by recording the bundle motion with a CCD camera (Qimaging; Rolera MXi) and an image registration method (33, 34). The mechanical stimulus was set to achieve a maximum amplitude plateau nqEPSC.

Time-resolved confocal microscopy (21) was used to examine the kinetics and spatial distribution of cleft acidification. The membrane impermeable indicator pyranine H348 (HPTS; P$K_+$ ~ 7.2, 20 μM; Invitrogen) was used; 500
swept field images (488 nm excitation, 488/568; Chroma Technology Detection) were collected at 2.5 frames s⁻¹ (Prairie, 63× 0.9 w; Zeiss Axioskop) while repeating stimuli at 0.2 or 0.3 c⁻¹. The spatially averaged fluorescence was fit with an exponential decay. The deviation away from the exponential provided ΔF and the value on the exponential provided F₀. Custom software time-stamped each pixel with the latency relative to the most recent stimulus trigger (21). Image data were analyzed using 1-μm² regions of interest (areas). A fluid jet stimulus was applied for ~50 periodic cycles, and Fourier analysis was applied.

To quantify the magnitude of pH changes, ratemeter membrane impermeant carboxy SNARF (pK = 7.5, 13 μM; Life Technologies) was used. A stiff glass probe was positioned to defocus a single hair cell ~1 μm in the excitatory direction using a 1-Hz square wave periodic stimulus.

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Zero Current Potential and Ionic Concentrations in the Cleft. Present data show that the nonquantal excitatory postsynaptic current (nqEPSC) was carried by $K^+$ and $Na^+$. Accounting for both ions, the Goldman–Hodgkin–Katz zero current potential $E_{nq}$ of the nqEPSC is given by

$$E_{nq} = \frac{RT}{F} \left( \ln \left( \frac{[K^+]_{120}}{1+P_{Na/K}} \right) + P_{Na/K} \ln \left( \frac{[Na^+]_5}{5} \right) \right),$$

where $R$ is the ideal gas constant, $T$ is absolute temperature, and $F$ is Faraday’s constant. $[K^+]$ and $[Na^+]$ (mM) are extracellular concentrations in the cleft, 120 and 5 mM are intracellular concentrations set by the recording pipette, and $P_{Na/K}$ is the permeability to $Na^+$ relative to $K^+$. It has been shown previously that $K^+$ entering the cleft from hair cells can increase cleft $[K^+]$ well beyond that of the extracellular space (1), and hence, concentrations in the extracellular media cannot be used to estimate concentrations in the cleft. To estimate $[K^+]$ and $[Na^+]$ concentrations in the cleft, we used zero current potentials in normal extracellular media and when extracellular $[Na^+]$ was replaced with $N$-methyl-$d$-glucamine (NMDG). Both $[K^+]$ and $[Na^+]$ in the cleft changed in the presence of NMDG. The zero current potential of the nqEPSC shifted from $E_{nq}$ in normal media to $E_{nq,}\text{NMDG}$ (mV) in NMDG, whereas there was little change in the magnitude of the nqEPSC. These results suggest that the total cationic concentration might be regulated in the cleft and that any gross decreases in $[Na^+]$ might be offset by increases in $[K^+]$. Under this assumption,

$$E_{nq} = \frac{RT}{F} \left( \ln \left( \frac{[K^+]_{120}}{1+P_{Na/K}} \right) + P_{Na/K} \ln \left( \frac{[T^+]-[K^+]_5}{5} \right) \right),$$

where $[T^+]$ is the total $[K^+]$ plus $[Na^+]$. To estimate concentrations, we assumed that $[T^+] = 125$ mM and minimized the cost function $\varepsilon = (E_{nq,\text{NMDG}} - 15)^2 + (E_{nq} - 44)^2$ numerically (using conservative estimates of $E_{nq}$ and $E_{nq,\text{NMDG}}$). This approach provided estimates of the relative permeability $P_{Na/K} = 5.2$ and ion concentrations (in the presence of NMDG: $[K^+]_{\text{NMDG, CLEFT}} = 115$ mM and $[Na^+]_{\text{NMDG, CLEFT}} = 10$ mM; concentrations in normal media: $[K^+]_{\text{CLEFT}} = 84$ mM and $[Na^+]_{\text{CLEFT}} = 41$ mM).

It is important to note that the voltage reported by the amplifier has an implicit error caused by the access series resistance. The error is small at the whole-cell zero-current potential (distinct from $E_{nq}$) but becomes large as the cell is depolarized by injection of current through the electrode. We used Eqs. S5 and S6 below to correct for it before plotting I–V curves and the nqEPSC reversal. The correction was relatively modest, because the whole-cell resistance was quite a bit larger than the electrode access series resistance.

Simple Model for Adapting $[H^+]$ Gated Channel. $[H^+]$ kinetics in the cleft were modeled using a nonlinear single compartment approach accounting for buffering and clearance as described in the text. The single compartment model mimics major features of $[H^+]$ buildup in the cleft observed in the present experiments. Conductance $G$ of the postsynaptic receptor was modeled as increasing with increased unbound free $[H^+]$ in the cleft (either directly or through a second messenger) according to

$$\frac{dG}{dt} = \frac{1}{\tau} (G_{max} - G) \left( 1 - q \right) h $$

where $\tau$ is the channel adaptation time constant, $0 \leq q \leq 1$ is the extent of adaptation, $g$ is the gain, and $h$ is the unit Heavyside function ($h = 1$ if $d[H^+]/dt \geq 0$; $h = 0$ otherwise). The nqEPSC was modeled as a chord conductance multiplied by the electrochemical driving potential

$$I = G(V_m - E_{nq}),$$

where $V_m$ is the membrane potential across the inner leaflet of the calyx facing the cleft, and $E_{nq}$ is the zero current potential of the nqEPSC given in Eq. S2. For rapid changes in pH, this model predicts a rapidly increasing current followed by adaptation, and for slow changes in pH, it predicts a slowly modulated current that tracks pH. In the present simulations, we used $\tau = 60$ ms, $q = 0.3, E_{nq} = 44$ mV, and $g_1 = 2 \times 10^{-4}$ $S \cdot m^{-1}$. Model equations are nonlinear, and therefore, results of simulations were fit with exponential curves to compare time constants with present experimental results.

Several shortcomings of this model are noted. (i) $[H^+]$ kinetics were modeled using a single compartment model and therefore, cannot describe large-scale spatial variations in $[H^+]$ that were shown to occur in the calyx (Fig. 4) or hypothetical microscale variations that could occur near clusters of channels. (ii) The model assumes a constant $H^+$ clearance rate $k$, which in reality, is likely to be voltage- and concentration-dependent. (iii) Eq. S3 describing the receptor is strictly empirical and does not account for any nonlinearity of the postsynaptic current. The single degree-of-freedom simplification (one time constant in Eq. S2) clearly prevents the model from capturing the change in time constant occurring near the reversal potential (Fig. 3A).

Series Resistance Correction. Electrode and whole-cell capacitance compensation was applied during the recording session. Series resistance was compensated after the experiment using custom software (IgorPro; WaveMetrics). Assuming ideal capacitance compensation and no series resistance compensation, the corrected membrane conduction current $I_M$ was approximated using

$$I_M \approx I_{EC}(1 + R_E/R_M) + C_M R_E \frac{dE_{EC}}{dt},$$

where $I_{EC}$ is the amplifier current recorded with capacitance compensation, $R_E$ is the electrode access resistance, $R_M$ is the whole-cell membrane resistance, and $C_M$ is the total capacitance (including membrane and electrode). The corresponding corrected membrane potential was approximated using

$$V_M \approx V_{EC} - R_E I_{EC},$$

where $V_{EC}$ is the capacitance-compensated voltage output by the amplifier. To derive these equations, we assumed that voltage-dependent dynamic responses caused by the series resistance-associated loss of voltage clamp were small. These equations were applied in the time domain before data analysis. Because of their slow kinetics, the capacitive component of the correction in Eq. S5 has no effect on the nqEPSCs, but the series resistance correction does have an effect and acts on both the current and the voltage (Eqs. S5 and S6).

pH Imaging. An animation of stimulus-evoked cleft acidification (Fig. 4) is provided in Movie S1. The animation shows stimulus-evoked acidification at ~70 points in the synaptic cleft based on...
Although the proportion of nqEPSCs was large, it is not known if protons directly gate the channel or if a more complex signaling pathway is at play. The channel permeability is consistent with common acid-sensing ion channels (ASICs), but the relatively slow and nonadapting kinetics suggest that the molecular mechanism is unlikely to reflect direct proton gating of a postsynaptic ASIC. Nevertheless, we investigated ASIC expression in our preparation to see if we could rule out this possibility.

Acid-Sensing Ion Channel Expression in the Calyx. Although the present results suggest that modulation of the nqEPSC is causal to changes in cleft pH, it is not known if protons directly gate the channel or if a more complex signaling pathway is at play. The channel permeability is consistent with common acid-sensing ion channels (ASICs), but the relatively slow and nonadapting kinetics suggest that the molecular mechanism is unlikely to reflect direct proton gating of a postsynaptic ASIC. Nevertheless, we investigated ASIC expression in our preparation to see if we could rule out this possibility.

Turtles were anesthetized and then perfused through the heart with heparinized (2 U/mL) PBS (0.01 M, pH 7.3) followed by 4% paraformaldehyde in PBS. In other immunostaining experiments, end organs from decapitated turtles were dissected and then immersion-fixed in 4% paraformaldehyde in PBS. After fixation, dissected end organs were rinsed, immersed in blocking buffer (5% normal goat serum in PBS with 0.05% Triton X-100 and 0.02% NaNO₃), and then incubated in primary antibody (3 µg/mL; mouse monoclonal anti-ASIC1; Millipore) and rabbit polyclonal anti-ASIC3 (3 µg/mL; BioSciences) diluted in PBS with 5% normal goat serum, 0.01% Triton X-100, and 0.02% NaNO₃ (incubation buffer). The samples were subsequently rinsed and incubated in fluorescent-tagged secondary reagents (goat anti-rabbit or anti-mouse IgG; Alexa dyes; Invitrogen) diluted in incubation buffer. Controls included end organs placed in incubation buffer without primary antibody or in an inappropriate secondary reagent (e.g., mouse monoclonal anti-ASIC1 followed by goat anti-rabbit IgG). No specific labeling was observed in these controls. All specimens were imaged with an Olympus Fluoview FV1000MPE multiphoton microscope with a Coherent Chameleon Vision II laser tuned to 780 nm and a 25x, 1.05-N.A. immersion objective.

Specific staining for ASIC1 and ASIC3 was observed in association with calyceal endings in both the lagena and the crista ampullares of the anterior and posterior semicircular canals. This labeling included both diffuse immunofluorescence throughout the calyx and small bright puncta located within the calyceal ending. It is not currently known if these channels play a role in the protonergic synaptic transmission reported here, but it seems likely that we cannot rule out this possibility. The bright puncta are likely to reflect clusters of ASIC channels within discrete regions of the calyx, but it is not known if they colocalize with sites of presynaptic proton extrusion (Fig. 4).

Morphology. We visualized the morphology of individual afferents and calyx terminals by filling the patch pipette with Lucifer yellow. Wide-field epifluorescence was used during the electrophysiological recording session, and in some cases, the tissue was fixed and visualized using confocal microscopy. Morphologies of calyces in the turtle lagena were similar to those reported previously in the turtle crista (3). Examples are provided in Fig. S3. Calyx-only afferents contact a single or multiple type I hair cells (three shown) (Fig. S3A). Dimorphic afferents with calyces envelop type I hair cells, and bouton terminals contact type II hair cells (three shown) (Fig. S3 B and C). In some cases, the dye entered the hair cell, indicating a breach of the inner membrane—these cases were excluded from the present study (Fig. S3C).

A. Extracellular pH Indicator

B. Intracellular pH Indicator

**Fig. S1.** The pH indicator ratiometric membrane impermeant carboxy SNARF was introduced into (A) the extracellular media through the bath or (B) the calyx through the voltage-clamp recording pipette. In all cases, the SNARF solution was pH-balanced to match SNARF-free solutions. (A, Left) Image showing time-averaged extracellular fluorescence around a complex calyx enveloping multiple type I hair cells and (A, Right) kinetics of spatially averaged extracellular fluorescence modulation ($\Delta F/F$) in response to a fluid jet-driven hair bundle displacement. (B, Left) Image showing time-averaged intracellular fluorescence within a simple calyx enveloping a single type I hair cell and (B, Upper Right) kinetics of spatially averaged intracellular fluorescence modulation ($\Delta F/F$) in response to fluid jet-driven hair bundle displacement. Intracellular ($\Delta F/F$) in the postsynaptic calyx was not detectable by the present methods and at least an order of magnitude smaller than $\Delta F/F$ in the extracellular cleft. (B, Lower Right) nqEPSCs were normal in the presence of the SNARF indicator, showing that the nqEPSC did not acidify the postsynaptic calyx.

**Fig. S2.** Immunofluorescence visualization of ASIC1 and ASIC3 in turtle end organs. (A) Both punctate and diffuse labeling are associated with calyces in the lagena. The boxed region is shown at a higher magnification in Inset. (B) The diffuse staining is present in the calyceal cytoplasm. (C and D) ASIC staining in the turtle canal cristae. Punctate (arrows) and diffuse (feathered arrows) immunolabeling for both channels are associated with calyceal endings.
Fig. S3. Calyx morphology. The voltage-clamp patch pipette was filled with Lucifer yellow to visualize the calyx and associated projections. In all cases, cells were patched on the outer leaflet of the calyx terminal adjacent to one of the type I hair cells enclosed by the calyx. The mechanical stimulus was positioned to deflect the type I hair bundle associated with the enclosed hair cell. (A) Calyx only and (B and C) dimorphic units are shown.

Movie S1. Stimulus-evoked acidification of the vestibular hair cell–calyx synaptic cleft. Background image shows time-averaged fluorescence of the pH indicator impermanent indicator pyrannine H348 added to the media and accumulated in the extracellular space around hair cells viewed perpendicular to the long axis of the cells. A single calyx envelops four hair cells. Synaptic clefts surrounding three hair cells are indicated by circular markers. The hair bundle of one hair cell was deflected with a 0.3-Hz fluid jet as indicated by the yellow bar. The size of each marker indicates the magnitude of the pH change directly under the marker. The largest pH change was around the stimulated hair cell and reached approximately −0.2 pH units. Changes around adjacent hair cells within the same calyx were smaller and time-delayed.

Movie S1