ATP release through connexin hemichannels and gap junction transfer of second messengers propagate Ca^{2+} signals across the inner ear

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Extracellular ATP controls various signaling systems including propagation of intercellular Ca^{2+} signals (ICS). Connexin hemichannels, P2x7 receptors (P2x7Rs), pannexin channels, anion channels, vesicles, and transporters are putative conduits for ATP release, but their involvement in ICS remains controversial. We investigated ICS in cochlear organotypic cultures, in which ATP acts as an IP_3-generating agonist and evokes Ca^{2+} responses that have been linked to noise-induced hearing loss and development of hair cell-afferent synapses. Focal delivery of ATP or photostimulation with caged IP_3 elicited Ca^{2+} responses that spread radially to several orders of unstimulated cells. Furthermore, we recorded robust Ca^{2+} signals from an ATP biosensor apposed to supporting cells outside the photostimulated area in WT cultures. ICS propagated normally in cultures lacking either P2x7R or pannexin-1 (Px1), as well as in WT cultures exposed to blockers of anion channels. By contrast, Ca^{2+} responses failed to propagate in cultures with defective expression of connexin 26 (Cx26) or Cx30. A companion paper demonstrates that, if expression of either Cx26 or Cx30 is blocked, expression of the other is markedly downregulated in the outer sulcus. Lanthanum, a connexin hemichannel blocker that does not affect gap junction (GJ) channels when applied extracellularly, limited the propagation of Ca^{2+} responses to cells adjacent to the photostimulated area. Our results demonstrate that these connexins play a dual crucial role in inner ear Ca^{2+} signaling: as hemichannels, they promote ATP release, sustaining long-range ICS propagation; as GJ channels, they allow diffusion of Ca^{2+}-mobilizing second messengers across coupled cells.

deafness | mouse models | P2x7 receptor | pannexin | biosensor cells

In the organ of Corti, a polarized neurosensory epithelium of the inner ear (1) [supporting information (SI) *Text* and Fig. S1], non-sensory epithelial and supporting cells form a glial-like syncytium (2) interconnected by connexin 26 (Cx26) and Cx30, two connexins that may assemble to form heteromeric gap junction (GJ) channels (3). In several systems coupled by GJ channels, including retina glial cells and astrocytic networks, the spread of intercellular Ca^{2+} signals (ICS) provides a mechanism by which cooperative cell activity is thought to be coordinated (4, 5). Nanomolar levels of ATP on the apical surface of the organ of Corti, which have been linked to sound exposure (6), activate G protein-coupled $P2Y_2$ and $P2Y_4$ receptors (7, 8). Moreover, focal mechanical stimuli that release ATP evoke IP_3-dependent ICS that propagate radially across this cochlear cellular network at a uniform and constant speed of 10 to 15 μm/s (7, 8), comparable to the speed of glial Ca^{2+} waves (4, 5). ICS propagation across the organ of Corti is reduced by apyrase, suramin, or intracellular acidification in CO_2-saturated buffer (7, 9). The mechanisms that underlie cochlear ICS propagation (7–9), as well as spontaneous ATP release in the Kölliker organ before the onset of hearing (10), are consistent with Ca^{2+}-activated ATP release through unpaired connexons (11), i.e., non-junctional Cx hemichannels (12, 13). Indeed, increase of cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) triggers Cx hemichannel opening (14, 15), and functional studies in expression systems indicate that Cx26 and Cx30 may operate as hemichannels in the plasma membrane (16–18). Furthermore, ATP is released in an actin- and phospholipase-C-dependent manner through Cx hemichannels in HeLa cell cultures stably expressing human Cx26 (19). Finally, deafness-linked mutations of Cx26 that result in abnormally open hemichannels cause cell death (20), whereas unregulated ATP release through Cx30 hemichannels has been implicated in alteration of epidermal factors leading to a rare skin disorder (21).

However, it has been pointed out that most studies implicating Cx hemichannels relied on the use of pharmacological compounds that are not specific and have been shown to affect the activity of various other channels (22). Therefore, alternative conduits for ATP release, like the P2x7 receptor (P2x7R) (23, 24), which is expressed in the immature inner ear (25), and its co-immunoprecipitating partner Px1 (26), cannot be excluded based solely on responses to pharmacological inhibitors or mimetic peptides (22, 27). Matters are further complicated by the fact that ICS can be also transmitted by the direct transfer of Ca^{2+}-mobilizing second messengers from the cytosol of one cell to that of an adjacent one through GJ channels (28). Indeed, GJ-mediated transmission of Ca^{2+} waves was the first pathway identified in astrocytes (29). Interestingly, ICS propagation across heteromeric GJ channels consisting of Cx26 and Cx30 is reported to be faster than across their homomerically assembled counterparts (30). Furthermore, heteromeric mixing of different connexin isoforms, producing variation in channel stoichiometry and/or arrangements of isoforms within the hemichannels, may allow cells to

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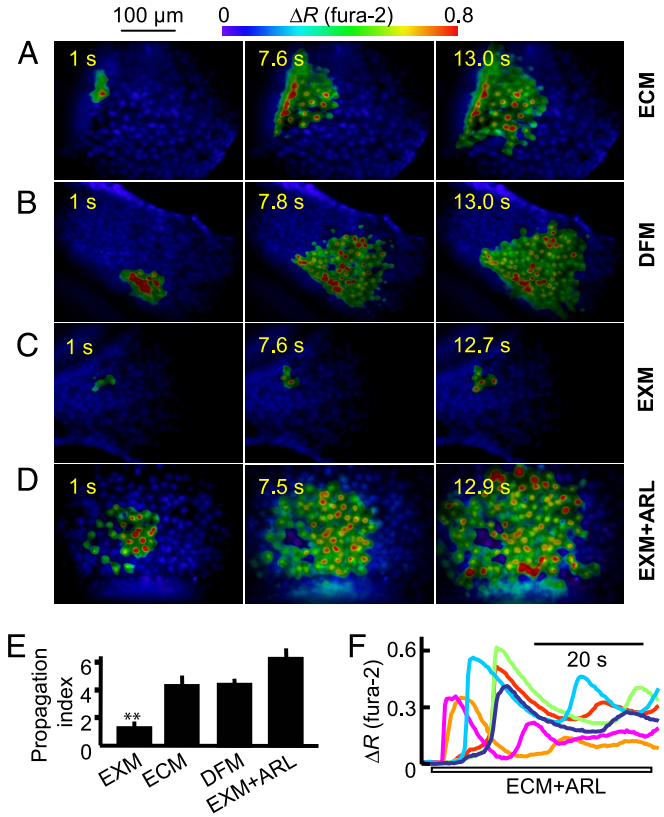

Fig. 1. Effect of divalent ions and ectonucleotidases on ICS evoked by ATP puffs in the organ of Corti. (*A-D*) Representative experiments showing pseudocolor images of Ca^{2+} signals (measured as fura-2 emission ratio changes; ΔR) propagating through cochlear supporting and epithelial cells of P3-P6 mouse organotypic cultures following focal delivery of an ATP puff (50 ms, 4 μM) from a glass micropipette with a tip opening of $\approx$1.5 μm. EXM, extracellular medium containing 2 mM Ca^{2+}; ECM, medium containing endolymphatic Ca^{2+} concentration (20 μM); DFM, medium devoid of divalent ions; EXM+ARL, ectonucleotidase inhibitor ARL67156 (100 μM) dissolved in EXM. Time of image capture measured from the end of ATP delivery is indicated on each frame. (*E*) Pooled data showing ICS propagation index for conditions in *A-D*; data are mean $\pm$ SEM for independently repeated experiments on at least five cultures from different animals; $^{**}P < 0.01$, Student *t* test. (*F*) Representative whole-cell averages of fura-2 ΔR versus time for randomly selected cells in a culture bathed in ECM and stimulated with 100 μM ARL67156 (empty bar at bottom marks timing of drug delivery).

regulate their intercellular communication properties, including molecular selectivity to inositol phosphates (31). Permeability to IP_3 is impaired in a deafness mutant of Cx26 (9).

By using focal ATP delivery (8) or photostimulation with caged IP_3 (32), we have performed accurate measurements of cochlear ICS propagation range, hereby defined as the ratio between the culture area invaded by Ca^{2+} signals and the area responding within 1 s to the applied stimulus (i.e., ATP puff or laser radiation). A value of 1 indicates no propagation (see *SI Text*). We show that Ca^{2+} signals fail to propagate in cultures with deficient expression of Cx26 and Cx30, whereas ICS in cultures from mice lacking Px1 or P2x7R are indistinguishable from those of WT controls.

Vesicular- and transporter-mediated release was not investigated in this study, as (*i*) vesicular storage of ATP has been detected in the cochlear lateral wall but not in cells of the organ of Corti (6, 33), and (*ii*) previous work has shown that blockage of either vesicular ATP release or ATP-binding cassette transporters fails to inhibit ATP release (11).

Results

Divalent Ions and Ectonucleotidases Control Ca^{2+} Signaling in WT Cochlear Cultures. In the first set of experiments (Fig. 1), we loaded cochlear organotypic cultures with the AM ester moiety of the Ca^{2+} indicator fura-2 and measured ICS in supporting and epithelial cells stimulated with ATP puffs (50 ms, 4 μM) delivered from a glass micropipette located above the outer hair cell region (Fig. S1) as previously described (7, 8). *In vivo*, the apical surface of the cochlear sensory epithelium is bathed in endolymph, an unusual extracellular fluid containing 150 mM K^+, 2 mM Na^+, and as little as 20 μM extracellular free Ca^{2+} concentration ($[Ca^{2+}]_o$) (34). We tested ICS propagation in ECM, a medium with endolymph-like $[Ca^{2+}]_o$ (Fig. 1*A* and Movie S1), or DFM, a medium devoid of divalent ions (Fig. 1*B*), and in both cases we obtained ICS similar to those evoked by focal mechanical stimuli (7, 8). ICS range decreased significantly in EXM, an extracellular medium containing 2 mM $[Ca^{2+}]_o$ (Fig. 1*C*). However, in EXM supplemented with the ectonucleotidase inhibitor ARL67156 (100 μM), the ICS range was similar to that measured in ECM (Fig. 1*D*; pooled data are shown in Fig. 1*E*), suggesting that EXM presumably reduces hemichannel basal opening, but the ATP that is released in the presence of ARL67156 is enough to support ICS propagation. Consistent with this hypothesis, in ECM supplemented with ARL67156 (100 μM), we recorded the spontaneous (i.e., unstimulated) occurrence of Ca^{2+} oscillations (Fig. 1*F*) similar to those elicited by extracellular ATP in concentrations ranging between 20 nM and $\approx$1 μM (7, 8). These results highlight the sensitivity of cochlear supporting cell Ca^{2+} signaling to $[Ca^{2+}]_o$ and ATP degradation by ectonucleotidases expressed at the endolymphatic surface of the organ of Corti (35, 36).

Ca^{2+} Signals Fail to Propagate in Connexin-Deficient Cochlear Cultures. By using antibodies against Cx26 extracellular loop peptides (18), we detected immunofluorescence signals near the apex of fixed, but not permeabilized, cochlear supporting and epithelial cells (Fig. S2), suggesting that unpaired connexons may be present in the apical plasma membrane, which prompted us to further test the hypothesis that Cx hemichannels release ATP in this system.

Thus, in the second set of experiments (Fig. 2), we co-loaded cochlear organotypic cultures from WT or transgenic mice with the AM esters of fura red (a visible light-excitable fura-2 analogue) and caged IP_3. By focusing UV laser light (100 ms, 375 nm) within a well defined area in the organ of Corti bathed in ECM, we photostimulated supporting cells containing caged IP_3, effectively bypassing the initial activation of P2Y receptors by extracellular ATP. In WT cultures, focal IP_3 uncaging elicited ICS that spread radially to several orders of unirradiated cells (Fig. 2*A* and Movie S2). ICS propagation was hampered in cultures lacking Cx26 (Fig. 2*B* and Movie S3) or Cx30 (Fig. 2*C*), but normal in cultures lacking either P2x7R (Fig. 2*D*) or Px1 (Fig. 2*E*). Pooled data are shown in Fig. 2 *F* and *G*. Sensitivity of Ca^{2+} responses to $[Ca^{2+}]_o$ and ectonucleotidases was similar in WT, P2x7R KO, and Px1 KO cultures (Fig. S3). Furthermore, the purinergic signaling cascade (7, 8) was intact in these experiments, as all cultures responded to extracellular ATP with the expected pattern of Ca^{2+} oscillations (not shown). The selective P2x7R inhibitor brilliant blue G and anion channel inhibitors SITS, DIDS, tamoxifen, and glybenclamide had no effect on ICS propagation in ECM (Fig. S4*A*), whereas ICS spread was inhibited in ECM supplemented with the nonselective Cx channel blockers CBX (in a dose-dependent manner), niflumic acid (200 μM), and flufenamic acid (50 μM) (Fig. S4*B*). Taken together, these experiments clearly show that connexins are involved in the process of ICS propagation illustrated in Fig. 2.

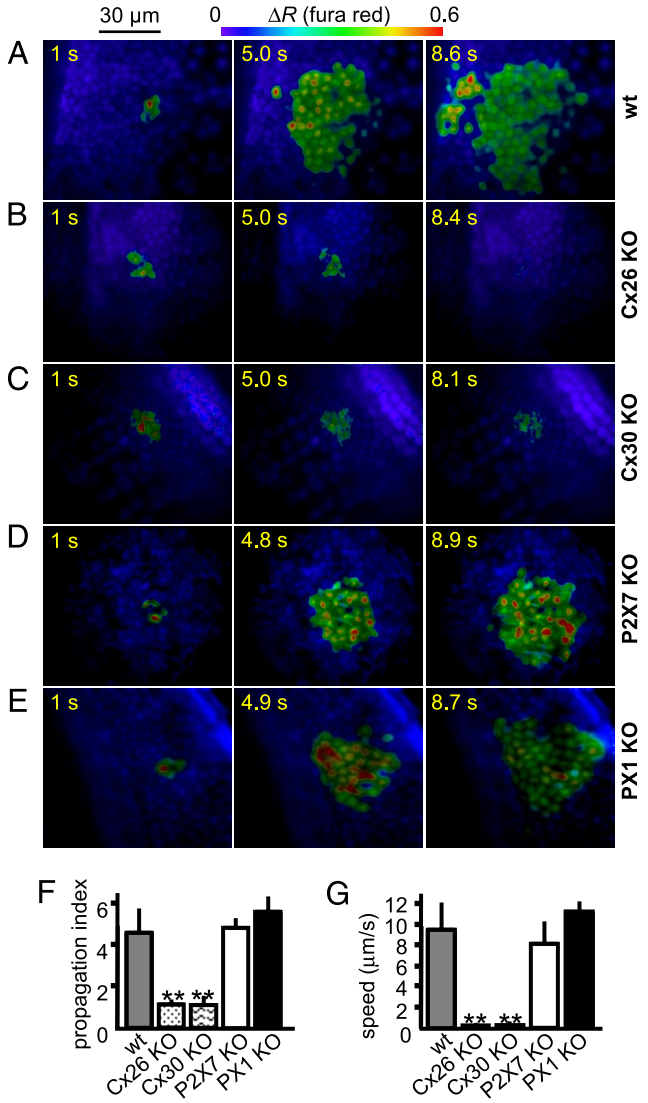

Fig. 2. Ca^{2+}signals evoked by 100 ms of focal photostimulation with caged IP_3 fail to propagate in connexin-deficient cochlear cultures but propagate normally in Px1 KO and P2x7 KO cultures. (*A–E*) Ca^{2+} signals in cochlear supporting and epithelial cells from P3-P6 mouse organotypic cultures bathed in ECM and co-loaded with the AM esters of fura red and caged IP_3. (*A*) Control (WT culture); (*B*) Cx26 KO culture; (*C*) Cx30 KO culture; (*D*) P2x7 KO culture; (*E*) Px1 KO culture. (*F*) Pooled data showing propagation range in WT controls and in cultures lacking Cx26, Cx30, P2x7, or Px1. (*G*) Speed of propagating ICS for data in *F*. Data in *F* and *G* are mean ± SEM for independently repeated experiments in tissues from at least three different animals.

Photostimulation with Caged IP_3 Releases ATP to the Extracellular Medium. To investigate the relationship between photorelease of caged IP_3, ATP release, and Cx hemichannels, we performed a third set of experiments using cellular biosensors (37) (Fig. 3). ATP biosensor responses are mediated by Ca^{2+} influx through P2x2 and P2x3 ionotropic receptors (37); therefore, data were collected in an extracellular medium containing 100 μM Ca^{2+}, a concentration that does not impair ICS propagation in WT cultures. We separately preloaded ATP biosensors with fluo-4AM and cochlear cultures with caged IP_3 AM. When a pipette-held ATP biosensor was apposed to a supporting cell outside the UV exposed area in a WT culture (Fig. 3 *A* and *B*), we reliably recorded robust biosensor Ca^{2+} signals (Fig. 3*C*). We failed to detect similar signals following IP_3 uncaging in Cx26 KO or Cx30 KO cultures (not shown). We note in passing that ICS propagated to cells in the Kölliker organ following IP_3 uncaging in Hensen cells of WT cultures bathed in ECM (Fig. S5 and Movie S4). Thus, ATP release through Cx hemichannels allows communication across supporting cells in the two separate GJ-coupled compartments (38) that are found on opposite sides of the sensory hair cell region, as well as within each one of these compartments.

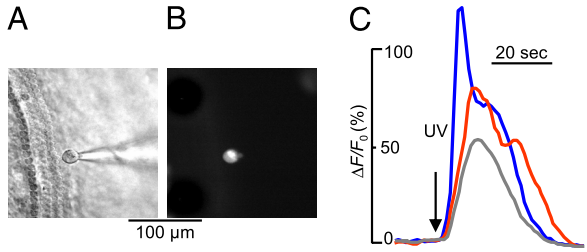

Fig. 3. Ca^{2+}responses evoked in ATP biosensors by 100 ms of focal photostimulation of cochlear supporting cells with caged IP_3. (*A*) Example of a pipette-held biosensor loaded with fluo-4 and apposed to a Hensen's cell in a culture loaded with caged IP_3 AM and viewed with bright-field differential interference contrast optics. (*B*) The same field under conventional epifluorescence illumination. (*C*) Representative biosensor whole-cell pixel averages of percent fluo-4 fluorescence change (ΔF) relative to pre-stimulus fluorescence emission intensity (F_0) measured before delivery of the photostimulation (arrow, stimulus onset), versus time for similar independently repeated experiments performed in tissues from three different animals.

Diffusion and Efflux of Calcein from Cochlear Supporting Cells. As cochlear Cx hemichannels release ATP, a charged and relatively large molecule (MW 340, charge −4 at physiological pH [39]), they might also release fluorescent tracers with similar charge and size. Calcein, which has approximately six negative and two positive charges at pH 7 (MW 622, net charge −4) is a polyanionic fluorescein derivative that, according to the manufacturer, exhibits fluorescence essentially independent of pH between 6.5 and 12. Thus, in the fourth set of experiments (Fig. 4 and Figs. S6–S8), we initially verified that calcein diffuses across GJ channels in the organ of Corti of WT cochlear cultures (Fig. S6). Thereafter, we assayed calcein efflux from cochlear cultures bathed in ECM and preloaded with the AM moiety of this tracer (Fig. 4). In particular, we compared calcein efflux rates in WT, Cx26 KO, and Cx30 KO cultures and we also tested the effects of CBX and lanthanum (La^{3+}). The latter has been reported to block IP_3-triggered ATP release in a rat brain endothelial cell line (40). La^{3+} also impairs dye uptake in rat cortical astrocytes expressing Cx43 (41) and blocks hemichannel currents evoked by depolarization of HeLa cells transfected with cDNA encoding rat Cx43 (42). We recorded hemichannel currents evoked by

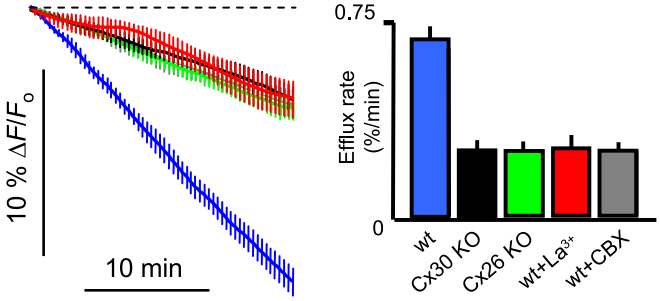

Fig. 4. Calcein efflux from supporting cells of the organ of Corti. *Left*, time course of percent fluorescence emission change (ΔF) relative to initial fluorescence intensity (F_0) in cultures preloaded with calcein AM. *Right*, efflux rate from data at left. Values shown as mean ± SEM for independently repeated experiments on cultures from at least four different animals.

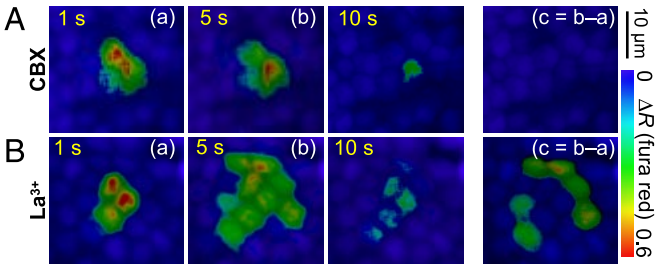

Fig. 5. Effects of CBX and La^{3+} on the spread of Ca^{2+} signals evoked by 100 ms of photostimulation with caged IP_3 in the organ of Corti. Ca^{2+} responses of cultures incubated with 100 μM CBX (*A*) or 100 μM La^{3+} (*B*). The fourth image in each sequence is the pixel-by-pixel difference (i.e., c = b − a) of the first two images. Results are representative of similar independently repeated experiments performed in tissues from at least three different animals.

depolarization in patch-clamped solitary HeLa cells transiently transfected with mouse Cx30 (16) tagged with a color variant of the GFP (43), and we determined that La^{3+} (100 μM) blocks these currents (Fig. S8). Addition of La^{3+} (100 μM) to ECM markedly reduced calcein efflux from WT cochlear cultures (Fig. 4). Indeed, in WT cultures exposed to La^{3+} (100 μM) or CBX (100 μM), calcein efflux rate was comparable to that of Cx26 KO or Cx30 KO cultures bathed in ECM but not exposed to any drug (Fig. 4). The residual efflux in the connexin KOs and in La^{3+} or CBX may be a result of dye loss (which may be tracer-dependent) through the membrane or other uncharacterized pathways. However, in gap-fluorescence recovery after photobleaching assays (44), calcein fluorescence recovery after photobleaching in WT cultures exposed to La^{3+} (100 μM) was similar to controls, whereas no recovery was observed in WT cultures exposed to CBX (100 μM; Fig. S7). Fluorescence recovery after photobleaching occurs by dye permeation through cell-cell channels, which partially restores the dye's intracellular pool within the photobleached cells. Incomplete recovery of fluorescence intensity is ascribed to the fraction of the pool that is not available for intercellular transfer, as a result of trapping into subcellular organelles and/or binding to subcellular structures (45). Thus, our experiments (Fig. 4 and Figs. S6–S8) clearly indicate that membrane-permeable CBX blocks both Cx hemichannels and GJ channels, whereas La^{3+} blocks Cx hemichannels but has no access to intercellular channels in GJ plaques. In summary, based on the experiments described so far and our previous work (7, 8), we conclude that Ca^{2+} signals can be transmitted across cochlear supporting and epithelial cells via extracellular diffusion of a signaling molecule, ATP, which is released through Cx hemichannels and acts on G protein-coupled $P2Y_2$ and $P2Y_4$ receptors (7, 8).

Diffusion of Ca^{2+}-Mobilizing Second Messengers Through GJ Channels. To investigate the relationship between ICS propagation and GJ channels, in the last series of experiments we exploited the different blocking characteristics of CBX and La^{3+} highlighted earlier. Thus, in cultures bathed in ECM co-loaded with caged IP_3 and fura red, uncaging IP_3 in the presence of CBX (100 μM) evoked Ca^{2+} signals that remained confined within the UV-irradiated area (Fig. 5*A*). In the presence of La^{3+} (100 μM), Ca^{2+} signal spread to first-order cells, i.e., cells adjacent to the irradiated area (Fig. 5*B*). The results in Fig. 5*B* are consistent with those obtained by directly injecting IP_3 in a single supporting cell while inhibiting P2Y receptors with suramin (9). Thus, we ascribe the spread of Ca^{2+} signals in Fig. 5*B* to the direct diffusion of Ca^{2+}-mobilizing second messengers, most likely IP_3 (9), through GJ channels.

Discussion

In this article we investigated the mechanisms underlying ICS propagation across epithelial and supporting cells of the organ of Corti challenged with extracellular ATP puffs or photostimulated with caged IP_3. Non-sensory cells of the cochlear epithelium express the intermediate filament glial fibrillary acidic protein, a classic marker for astrocytes (2), and are thought to perform similar spatial buffering of K^+, the major charge carrier implicated in mechano-electrical transduction (3, 46, 47). Activation of Cx43 hemichannels in the astrocyte plasma membrane causes the release of ATP into the extracellular space (48), where it activates P2Y receptors on adjacent cells to initiate a Ca^{2+} wave (12, 13).

In addition, Px1 and P2x7R have been implicated in ATP secretion from glial cells during Ca^{2+} wave propagation (49) and amplification of astrocytic ICS (24). Px1 can assemble in the plasma membrane (50, 51), forming mechano-sensitive conduits that are permeable to ATP (52) and underlie ATP release from erythrocytes challenged by mechanical stretch (53), as well as mouse taste receptors activated by gustatory stimuli (37). However, determining the exact function for pannexons and how they differ from connexons has been so far undermined by lack of specific channel blocker and Px gene KO animals (54). Furthermore, interactions between Px1 and P2x7R have been documented. In particular, co-expression of Px1 and P2x7R produces a signaling complex that, when activated by ATP, leads to opening of a cation channel, the creation of a transmembrane pathway sufficiently large to allow the passage of large molecular weight fluorescent dyes (up to 900 Da), and cell lysis (26, 55). The large-pore permeation properties of the P2x7R/Px1 complex are greatly enhanced by removal of divalent cations and inhibited by CBX (55) whereas, in the absence of Px1, P2x7R behaves like a simple cation channel that is resistant to treatment with CBX and other nonselective connexin channel blockers (26, 55, 56).

Our results with caged IP_3 and genetically targeted mice overcome ambiguities resulting from a lack of specific channel blockers, demonstrating that ICS propagate normally in cultures lacking either P2x7R or Px1, but fail to propagate in cultures with defective expression of Cx26 or Cx30. We thus exclude that channels composed of Px1, P2x7R, or a combination of the two play a significant role. Besides P2x7R (25), the only P2X-type receptor that has been detected consistently in epithelial, supporting, and sensory cells of the rat organ of Corti is the P2x2R (57). In the developing cochlea, at P6 (an age pertinent to our studies), Hensen's cells, Deiters' and inner phalangeal cell processes, and Claudius cells, all express P2x2R (57). However, we did not detect significant differences in ICS propagation using DFM or ECM, consistent with the fact that ATP-gated ion currents recorded from cochlear supporting cells activate at ATP concentrations in excess of 10 μM (58). By contrast, based on the comparison of our fura-2 Ca^{2+} signals in Fig. 1 to the ATP dose-response curve in figure 2 of Gale et *al.* (7), we estimate that ATP levels stay in the sub-micromolar range during ICS propagation in the organ of Corti. Hence we exclude P2x2R contributions to the Ca^{2+} signals presented in this article.

Anion channel inhibitors had no effect on ICS propagation in ECM (Fig. S4A), whereas, as mentioned earlier in this article, contributions from vesicular- and transporter-mediated ATP release can be excluded based on previous work (11). Therefore, based on evidence presented in this article and in our previous work (7–9), we conclude that connexins afford two concurrent pathways by which ICS propagate across the organ of Corti; one involves release of ATP through Cx hemichannels and its diffusion in the extracellular space, coupled to activation of $P2Y_2$ and $P2Y_4$ membrane receptors that are responsive to nanomolar ATP in this system (7, 8); the other involves the transfer of

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Ca^{2+}-mobilizing signaling molecules, and possibly also other types of second messengers (59), directly from the cytosol of one cell to that of an adjacent cell through GJ channels (9).

The unique composition of endolymph makes ATP release through Cx hemichannels an attractive candidate for ICS propagation *in vivo*, as low endolymphatic $[Ca^{2+}]_o$ (34) is expected to bias Cx hemichannels in the apical plasma membrane toward the open state (60). Another important factor that may contribute to enhancing hemichannel activity in cells exposed to endolymph is the high level of endolymphatic K^+ (61). Frequency and duration of Cx hemichannel opening may be additionally regulated by physiological intracellular triggers, most notably intracellular Ca^{2+} (14, 15). Thus, work performed on Cx32-expressing cells showed that an increase in $[Ca^{2+}]_i$ triggers ATP release (and dye uptake) that is critically dependent on $[Ca^{2+}]_i$ elevation within a concentration "window" situated around 500 nM (see figs. 7 *E* and *F* of ref. 14). Although we have not performed a similar characterization of $[Ca^{2+}]_i$-dependent ATP release, it is interesting to note that ICS mobilize $[Ca^{2+}]_i$ to peak levels of $\approx$500 nM in our system (7, 9).

Complete blockade of ICS propagation in cochlear cultures lacking either Cx26 or Cx30 is not entirely unexpected, as a substantial decrease in Cx26 protein level (but not mRNA) was recently reported in cochlea of Cx30 KO mice (62). Likewise, the amount of Cx26 protein in liver cells of Cx32 KOs is significantly lower than in controls (63). We analyzed this phenomenon in detail and, in a companion article, we provide evidence for coordinated regulation of inner ear Cx26 and Cx30 expression, at both the mRNA and the protein level, as a result of signaling through phospholipase C and NFκB pathway in cochlear supporting and epithelial cells in a region between the outer hair cells and the stria vascularis. However, other unexplored facets call for further analyses. Indeed, in our opinion, one of the main goals for future research is certainly that of achieving a clearer picture of intercellular signaling in the inner ear.

Materials and Methods

Organ Cultures. Cochleae were dissected from P3-P6 mouse pups in ice-cold Hepes-buffered (10 mM, pH 7.2) Hanks' balanced salt solutions (HBSS; Sigma) and placed onto glass coverslips coated with 10 g/ml of Cell Tak (Becton Dickinson), as described by Gale *et al.* (7). Cultures were incubated in DMEM/F12 (Invitrogen), supplemented with FBS 5% and maintained at 37 °C for 1 d. For experiments, cultures were superfused at 2 ml/min with an extracellular medium (i.e., EXM) containing 138 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.3 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 10 mM Hepes-NaOH, and 6 mM d-glucose (pH 7.2; 320 mOsm). ECM refers to a similar medium with a reduced Ca^{2+} concentration of 20 μM. DFM refers to a similar medium devoid of divalent ions. Experiments were performed at room temperature, 22 °C-24 °C. For further methodological details, see the *SI Text*.

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1. Krstic RV (1997) *Human Microscopic Anatomy*. (Springer-Verlag, Berlin).
2. Rio C, Dikkes P, Liberman MC, Corfas G (2002) Glial fibrillary acidic protein expression and promoter activity in the inner ear of developing and adult mice. *J Comp Neurol* 442:156–162.
3. Zhao HB, Kikuchi T, Ngezahayo A, White TW (2006) Gap junctions and cochlear homeostasis. *J Membr Biol* 209:177–186.
4. Metea MR, Newman EA (2006) Calcium signaling in specialized glial cells. *Glia* 54:650–655.
5. Scemes E, Giaume C (2006) Astrocyte calcium waves: what they are and what they do. *Glia* 54:716–725.
6. Munoz DJ, Kendrick IS, Rassam M, Thorne PR (2001) Vesicular storage of adenosine triphosphate in the guinea-pig cochlear lateral wall and concentrations of ATP in the endolymph during sound exposure and hypoxia. *Acta Otolaryngol* 121:10–15.
7. Gale JE, Piazza V, Ciubotaru CD, Mammano F (2004) A mechanism for sensing noise damage in the inner ear. *Curr Biol* 14:526–529.
8. Piazza V, Ciubotaru CD, Gale JE, Mammano F (2007) Purinergic signalling and intercellular Ca^{2+} wave propagation in the organ of Corti. *Cell Calcium* 41:77–86.
9. Beltramello M, Piazza V, Bukauskas FF, Pozzan T, Mammano F (2005) Impaired permeability to $Ins(1,4,5)P_3$ in a mutant connexin underlies recessive hereditary deafness. *Nat Cell Biol* 7:63–69.
10. Tritsch NX, Yi E, Gale JE, Glowatzki E, Bergles DE (2007) The origin of spontaneous activity in the developing auditory system. *Nature* 450:50–55.
11. Zhao HB, Yu N, Fleming CR (2005) Gap junctional hemichannel-mediated ATP release and hearing controls in the inner ear. *Proc Natl Acad Sci USA* 102:18724–18729.
12. Bennett MVL, Contreras JE, Bukauskas FF, Saez JC (2003) New roles for astrocytes: gap junction hemichannels have something to communicate. *Trends Neurosci* 26:610–617.
13. Goodenough DA, Paul DL (2003) Beyond the gap: functions of unpaired connexon channels. Nat Rev *Mol Cell Biol* 4:285–294.
14. De Vuyst E, *et al.* (2006) Intracellular calcium changes trigger connexin 32 hemichannel opening. *EMBO J* 25:34–44.
15. Pearson RA, Dale N, Llaudet E, Mobbs P (2005) ATP released via gap junction hemichannels from the pigment epithelium regulates neural retinal progenitor proliferation. *Neuron* 46:731–744.
16. Valiunas V, Weingart R (2000) Electrical properties of gap junction hemichannels identified in transfected HeLa cells. *Pflugers Arch* 440:366–379.
17. Gonzalez D, Gomez-Hernandez JM, Barrio LC (2006) Species specificity of mammalian connexin-26 to form open voltage-gated hemichannels. *FASEB J* 20:2329–2338.
18. Clair C, Combettes L, Pierre F, Sansonetti P, Tran Van Nhieu G (2008) Extracellular-loop peptide antibodies reveal a predominant hemichannel organization of connexins in polarized intestinal cells. *Exp Cell Res* 314:1250–1265.
19. Tran Van Nhieu G, Clair C, Bruzzone R, Mesnil M, Sansonetti P, Combettes L (2003) Connexin-dependent inter-cellular communication increases invasion and dissemination of Shigella in epithelial cells. *Nat Cell Biol* 5:720–726.
20. Stong BC, Chang Q, Ahmad S, Lin X (2006) A novel mechanism for connexin 26 mutation linked deafness: cell death caused by leaky gap junction hemichannels. *Laryngoscope* 116:2205–2210.
21. Essenfelder GM, *et al.* (2004) Connexin30 mutations responsible for hidrotic ectodermal dysplasia cause abnormal hemichannel activity. *Hum Mol Genet* 13:1703–1714.
22. Spray DC, Ye ZC, Ransom BR (2006) Functional connexin "hemichannels": a critical appraisal. *Glia* 54:758–773.
23. Pellegatti P, Falzoni S, Pinton P, Rizzuto R, Di Virgilio F (2005) A novel recombinant plasma membrane-targeted luciferase reveals a new pathway for ATP secretion. *Mol Biol Cell* 16:3659–3965.
24. Suadicani SO, Brosnan CF, Scemes E (2006) P2X7 receptors mediate ATP release and amplification of astrocytic intercellular Ca^{2+} signaling. *J Neurosci* 26:1378–1385.
25. Nikolic P, Housley GD, Thorne PR (2003) Expression of the P2X7 receptor subunit of the adenosine 5′-triphosphate-gated ion channel in the developing and adult rat cochlea. *Audiol Neurootol* 8:28–37.
26. Pelegrin P, Surprenant A (2006) Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. *EMBO J* 25:5071–5082.
27. Wang J, Ma M, Locovei S, Keane RW, Dahl G (2007) Modulation of membrane channel currents by gap junction protein mimetic peptides: size matters. *Am J Physiol Cell Physiol* 293:C1112–C1119.
28. Saez JC, Connor JA, Spray DC, Bennett MVL (1989) Hepatocyte gap junctions are permeable to the second messenger, inositol 1,4,5-trisphosphate, and to calcium ions. *Proc Natl Acad Sci USA* 86:2708–2712.
29. Finkbeiner S (1992) Calcium waves in astrocytes-filling in the gaps. *Neuron* 8:1101–1108.
30. Sun J, *et al.* (2005) Cochlear gap junctions coassembled from Cx26 and 30 show faster intercellular Ca^{2+} signaling than homomeric counterparts. *Am J Physiol Cell Physiol* 288:C613–C623.
31. Ayad WA, Locke D, Koreen IV, Harris AL (2006) Heteromeric, but not homomeric, connexin channels are selectively permeable to inositol phosphates. *J Biol Chem* 281:16727–16739.
32. Leybaert L, Sanderson MJ (2001) Intercellular calcium signaling and flash photolysis of caged compounds: a sensitive method to evaluate gap junctional coupling. *Methods Mol Biol* 154:407–430.
33. White PN, *et al.* (1995) Quinacrine staining of marginal cells in the stria vascularis of the guinea-pig cochlea: a possible source of extracellular ATP? *Hear Res* 90:97–105.
34. Bosher SK, Warren RL (1978) Very low calcium content of cochlear endolymph, an extracellular fluid. *Nature* 273:377–378.
35. Housley GD, *et al.* (2002) Purinergic regulation of sound transduction and auditory neurotransmission. *Audiol Neurootol* 7:55–61.
36. Vlajkovic SM, *et al.* (2004) Noise exposure induces up-regulation of ecto-nucleoside triphosphate diphosphohydrolases 1 and 2 in rat cochlea. *Neuroscience* 126:763–773.
37. Huang YJ, *et al.* (2007) The role of pannexin 1 hemichannels in ATP release and cell-cell communication in mouse taste buds. *Proc Natl Acad Sci USA* 104:6436–6441.

38. Jagger DJ, Forge A (2006) Compartmentalized and signal-selective gap junctional coupling in the hearing cochlea. *J Neurosci* 26:1260–1268.
39. Storer AC, Cornish-Bowden A (1976) Concentration of MgATP2- and other ions in solution. Calculation of the true concentrations of species present in mixtures of associating ions. *Biochem J* 159:1–5.
40. Braet K, *et al.* (2003) Pharmacological sensitivity of ATP release triggered by photoliberation of inositol-1,4,5-trisphosphate and zero extracellular calcium in brain endothelial cells. *J Cell Physiol* 197:205–213.
41. Contreras JE, *et al.* (2002) Metabolic inhibition induces opening of unapposed connexin 43 gap junction hemichannels and reduces gap junctional communication in cortical astrocytes in culture. *Proc Natl Acad Sci USA* 99:495–500.
42. Contreras JE, Saez JC, Bukauskas FF, Bennett MVL (2003) Gating and regulation of connexin 43 (Cx43) hemichannels. *Proc Natl Acad Sci USA* 100:11388–11393.
43. Beltramello M, *et al.* (2003) Permeability and gating properties of human connexins 26 and 30 expressed in HeLa cells. *Biochem Biophys Res Commun* 305:1024–1033.
44. Wade MH, Trosko JE, Schindler M (1986) A fluorescence photobleaching assay of gap junction-mediated communication between human cells. *Science* 232:525–528.
45. Rabut G, Ellenberg J (2005) Photobleaching techniques to study mobility and molecular dynamics of proteins in live cells: FRAP, iFRAP, and FLIP. *Live Cell Imaging*, ed Goldman RD, Spector DL (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY), pp 101–126.
46. Hibino H, Kurachi Y (2006) Molecular and physiological bases of the K+ circulation in the mammalian inner ear. *Physiology (Bethesda)* 21:336–345.
47. Wangemann P (2006) Supporting sensory transduction: cochlear fluid homeostasis and the endocochlear potential. *J Physiol* 576:11–21.
48. Kang J, *et al.* (2008) Connexin 43 hemichannels are permeable to ATP. *J Neurosci* 28:4702–4711.
49. Barbe MT, Monyer H, Bruzzone R (2006) Cell-cell communication beyond connexins: the pannexin channels. *Physiology (Bethesda)* 21:103–4114.
50. Bruzzone R, Barbe MT, Jakob NJ, Monyer H (2005) Pharmacological properties of homomeric and heteromeric pannexin hemichannels expressed in Xenopus oocytes. *J Neurochem* 92:1033–1043.
51. Boassa D, *et al.* (2007) Pannexin1 channels contain a glycosylation site that targets the hexamer to the plasma membrane. *J Biol Chem* 282:31733–31743.
52. Bao L, Locovei S, Dahl G (2004) Pannexin membrane channels are mechanosensitive conduits for ATP. *FEBS Lett* 572:65–68.
53. Locovei S, Bao L, Dahl G (2006) Pannexin 1 in erythrocytes: function without a gap. *Proc Natl Acad Sci USA* 103:7655–7659.
54. Shestopalov VI, Panchin Y (2008) Pannexins and gap junction protein diversity. *Cell Mol Life Sci* 65:376–394.
55. Locovei S, Scemes E, Qiu F, Spray DC, Dahl G (2007) Pannexin1 is part of the pore forming unit of the P2X(7) receptor death complex. *FEBS Lett* 581:483–488.
56. Petrou S, Ugur M, Drummond RM, Singer JJ, Walsh JV Jr (1997) P2X7 purinoceptor expression in Xenopus oocytes is not sufficient to produce a pore-forming P2Z-like phenotype. *FEBS Lett* 411:339–345.
57. Jarlebark LE, Housley GD, Raybould NP, Vlajkovic S, Thorne PR (2002) ATP-gated ion channels assembled from P2X2 receptor subunits in the mouse cochlea. *Neuroreport* 13:1979–1984.
58. Lagostena L, Ashmore JF, Kachar B, Mammano F (2001) Purinergic control of intercellular communication between Hensen's cells of the guinea-pig cochlea. *J Physiol* 531:693–706.
59. Hernandez VH, *et al.* (2007) Unitary permeability of gap junction channels to second messengers measured by FRET microscopy. *Nat Methods* 4:353–358.
60. Saez JC, Retamal MA, Basilio D, Bukauskas FF, Bennett MVL (2005) Connexin-based gap junction hemichannels: gating mechanisms. *Biochim Biophys Acta* 1711:215–224.
61. Srinivas M, Calderon DP, Kronengold J, Verselis VK (2006) Regulation of connexin hemichannels by monovalent cations. *J Gen Physiol* 127:67–75.
62. Ahmad S, *et al.* (2007) Restoration of connexin26 protein level in the cochlea completely rescues hearing in a mouse model of human connexin30-linked deafness. *Proc Natl Acad Sci USA* 104:1337–1341.
63. Nelles E, *et al.* (1996) Defective propagation of signals generated by sympathetic nerve stimulation in the liver of connexin32-deficient mice. *Proc Natl Acad Sci USA* 93:9565–9570.