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Hemichannel-Mediated Inositol 1,4,5-Trisphosphate (IP₃) Release in the Cochlea: A Novel Mechanism of IP₃ Inter cellular Signaling

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Abstract

Inositol 1,4,5-trisphosphate (IP₃) is an important second messenger that can trigger a Ca²⁺ wave prolonged between cells. This intercellular signaling was found defective in some gap junction connexin deafness mutants. In this study, the mechanism underlying IP₃ intercellular signaling in the cochlea was investigated. A gap junction channel is composed of two hemichannels. By using a fluorescence polarization technique to measure IP₃ concentration, the authors found that IP₃ could be released by gap junction hemichannels in the cochlea. The IP₃ release was increased about three- to fivefold by the reduction of extracellular Ca²⁺ concentration or by mechanical stress. This incremental release could be blocked by gap junction blockers but not eliminated by a purinergic P2x receptor antagonist and verapamil, which is a selective P-glycoprotein inhibitor inhibiting the ATP-binding cassette transporters. The authors also found that IP₃ receptors were extensively expressed in the cochlear sensory epithelium, including on the cell surface. Extracellular application of IP₃ could trigger cellular Ca²⁺ elevation. This Ca²⁺ elevation was eliminated by the gap junction hemichannel blocker. These data reveal that IP₃ can pass through hemichannels acting as an extracellular mediator to participate in intercellular signaling. This hemichannel-mediated extracellular pathway may play an important role in long-distance intercellular communication in the cochlea, given that IP₃ only has a short lifetime in the cytoplasm.

Keywords

calcium; gap junction; hearing loss; hemichannel; IP₃; intercellular communication

INTRODUCTION

The gap junction is an intercellular channel that forms an intracellular conduit allowing direct exchange of small molecules and ions between cells. A gap junction channel is formed by two hemichannels. Each hemichannel is composed of six subunits, which are hexagonally arranged, forming a relatively large pore (~10 Å) through which ions and small molecules up to 1 kDa can pass (Harris 2001). Recent studies have shown that the role

of hemichannels extends beyond the formation of gap junction channels between cells. They can function solely on the cell surface to construct an alternative communication pathway for extracellular space rather than intracellular cytoplasm between adjacent cells (Bennett et al. 2003; Goodenough and Paul 2003). This hemichannel communicative potency has been directly demonstrated by dye-uptake assays and the measurement of the release of cell signaling molecules, such as ATP, glutamate, and NAD^+ (Bruzzone et al. 2001; Contreras et al. 2002; Stout et al. 2002; Ye et al. 2003; Zhao et al. 2005). The opening of hemichannels can be mediated by Ca^{2+} and member tension (Harris 2001; Bennett et al. 2003; Goodenough and Paul 2003). Depending on conditions, the opening can be physiological or pathological (Kamermans et al. 2001; Zhao et al. 2005; Thompson et al. 2006).

Inositol 1,4,5-triphosphate (IP_3) is an important second messenger, whose primary function is to elevate intracellular Ca^{2+} concentration by activating IP_3 receptors to release Ca^{2+} from the intracellular store and to allow external Ca^{2+} influxing (Berridge 1993; Dellis et al. 2006). IP_3 can also evoke a Ca^{2+} wave propagated between cells via gap-junctional coupling (Niessen et al. 2000). Recently, it has been found that this IP_3 intercellular signaling may play an important role in hearing: some Cx26 deafness mutants impaired the propagation of IP_3 -evoked Ca^{2+} waves between cells (Beltramello et al. 2005). However, the IP_3 permeability and the mechanisms underlying the IP_3 intercellular signaling in the cochlea remain undetermined. In this study, by using the fluorescence polarization technique to directly measure the IP_3 concentration, we found that hemichannels in the cochlear sensory epithelium could release IP_3 under physiological conditions. We also found that the extracellular application of IP_3 could elevate the intracellular Ca^{2+} concentration. These data suggest that IP_3 can pass through hemichannels to participate in intercellular communication via an extracellular pathway in the cochlea.

MATERIALS AND METHODS

Cochlear Isolation and Incubation for Hemichannel-Releasing Measurement

The cochlea was freshly isolated from adult guinea pigs (250 to 450 g). Cochlear isolation and the procedure of measurement for hemichannel release have been fully described elsewhere (Zhao 2005a; Zhao et al. 2005). Briefly, the cochlea was microdissected in a sterile normal extracellular solution (ECS) (in mM: 142 NaCl, 5.37 KCl, 1.47 MgCl_2 , 2 CaCl_2 , 10 HEPES, 25 dextrose; osmolarity 300 mOsm and pH 7.2). After removal of the bone, the organ of Corti was isolated. Then, the isolated cochlea was incubated in 200 μl ECS in an incubation chamber. Every 5 min, the incubation solution (200 μl) was collected and replaced with fresh solution. After two collections, the cochlea was switched to be incubated with a sterile, nominally Ca^{2+} - and Mg^{2+} -free extracellular solution (in mM: 142 NaCl, 5.37 KCl, 10 HEPES, 25 dextrose; 300 mOsm and pH 7.2) for 15 min (three collections) to test hemichannel release. Then, the cochlea was returned back to incubation in the normal ECS and sampled three more times (see the trace at the bottom of Figure 2A). All experiments were performed at room temperature (23°C).

IP₃ Measurement by Fluorescence Polarization Technique

The amount of IP₃ was measured using a fluorescence polarization assay, which is based on competitive binding between an IP₃ fluorescence tracer and unlabeled IP₃ from the sample solution. Free IP₃ competes for binding to the IP₃-binding protein and allows the IP₃ tracer to rotate freely upon excitation with plain polarized light. The polarized signal (mP) is inversely proportional to the amount of the free unlabeled IP₃; high levels of free IP₃ result in fluorescence emission being depolarized (low mP) (Owicki 2000). The measurement was performed by a HitHunter fluorescence polarization assay kit (catalog number 90-0037; DiscoverX Tech, Fremont, CA). The incubating solution sample (20 μ l) was mixed with the reagents following the manufacturer's instructions. A black 96-well plate was used for measurement to avoid optical cross-talk between wells. The fluorescence emission was read by a Wallac 1420 Victor Multilabel Plate Reader (PerkinElmer, Wellesley, MA). The IP₃ concentration was calculated from the IP₃ standard curve, which was measured from threefold serial dilutions of a IP₃ standard solution (20 μ M) and fitted with a four-parameter Hill equation: $y = y_0 + a / (1 + (x / c)^b)$. The measured standard curve showed a high regression ($R=0.997$) (Figure 1). The data were compiled and presented as mean $\pm$ SD.

Mechanical Stimulation and Membrane Stress Application

Mechanical stimulation was performed by gently pipetting (once every 3 to 4 s) the incubated cochlea with 10 mg/ml micro glass beads ($\phi=30\text{--}50\text{ }\mu\text{m}$; Polysciences, Warrington, PA) in the normal ECS solution (Zhao et al. 2005). To apply membrane stress, the normal ECS (300 mOsm) was replaced with a hypotonic ECS (275 mOsm) and the same procedure described above was implemented to as replace 0 mM Ca²⁺ ECS. The osmolarity of all solutions was measured by a microcomputer-controlled osmometer (Model 3300; Advanced Instruments, Norwood, MA) and adjusted by dextrose.

Fluorescence Dye-Uptake Assay

A membrane-impermeable fluorescence dye Lucifer Yellow (LY; MW=444; charge -2), which has similar size and charge to IP₃, was used for dye-uptake assay. As previously reported (Zhao 2005a), cells or cochlear sensory epithelia were incubated with 1 mM LY in the Ca²⁺-free ECS for 10 min and then washed out by normal ECS. The cellular dye uptake was observed under a microscope (Nikon, TE300) with corresponding fluorescence filters. The image was captured by a CCD camera.

Ca²⁺ Fluorescence Imaging and Measurement

The cochlear sensory epithelium was incubated in normal extracellular solution with 5 μ M Fluo-4 AM (F-14217; Molecular Probes) for 30 min at room temperature. After washout and deesterification for 20 to 30 min, the intensity of the Fluo-4 fluorescence emission was continuously recorded online by a photometer (Photon Tech. Int., Birmingham, NJ) under a Nikon microscope. The fluorescence image was also captured by a CCD camera.

Applications of Gap Junction Blockers and BAPTA-AM

The gap junction channel blockers 18 α -glycyrrhetinic acid (18-AGA; 50 μ M), carbenoxolone (CBX; 0.1 mM), and octanol (10 mM) were used in the experiments. These

gap junction blockers are known to block hemichannel dye uptake in cochlear supporting cells (Zhao 2005a). The blocker was added into both Ca^{2+} -free and normal extracellular solutions (Zhao et al. 2005). For intracellularly loading of the calcium-chelating reagent BAPTA, the cochlea was preincubated in the ECS with 20 μM BAPTA-AM (B6769; Molecular Probes) for 45 min, followed by 20 min incubation in normal ESC for de-esterification.

Immunofluorescence Staining

The detailed method for immunofluorescence staining has been described in our previous report (Zhao and Yu 2006). The cochlear sensory epithelium or the dissociated cochlear cells were fixed with 4% paraformaldehyde for 30 min. After washout, the epithelium was incubated in a blocking solution (10% goat serum and 1% bovine serum albumin [BSA] in phosphate-buffered saline [PBS]) with 0.1% Triton X-100 for 20 min. The epithelium was then incubated with rabbit polyclonal anti-IP₃R (IP₃ receptor) antibody (1:100; sc-28163; Santa Cruz Biotechnology, Santa Cruz, CA) in the blocking solution at 4°C overnight. After washing out, the epithelium was allowed to react with the secondary antibody Alexa Fluor 488 (1:500; Molecular Probes) in the blocking solution, at room temperature for 1 h, to visualize staining. The stained epithelia or cells were observed under a Leica confocal microscope (Leica TCS SP2).

RESULTS

Hemichannel-Mediated IP₃ Release in the Cochlear Sensory Epithelium

The hemichannel-mediated IP₃ release was measured by use of a fluorescence polarization assay (Figure 2). As the cochlear sensory epithelium was incubated in nominally Ca^{2+} -free extracellular solution (ECS) (presented by the bottom trace in Figure 2A) to interfere with hemichannel openings, the IP₃ release was increased. The amount of IP₃ increased from $4.8 \pm 1.5 \times 10^{-13}$ to $14.6 \pm 3.8 \times 10^{-13}$ moles, almost a threefold increase (Figure 2A and B). The IP₃ concentration normalized to the guinea pig's cochlear space volume, which is about 10 μl (Thorne et al. 1999), was increased from 48 ± 15 nM to 0.15 ± 0.04 μM (Figure 2B). The level of IP₃ release returned to the baseline when the cochlea was reincubated in the 2 mM Ca^{2+} ECS. This incremental increase in IP₃ release could be blocked by application of the gap junction blockers carbenoxolone (CBX; 0.1 mM), 18 α -glycyrrhentic acid (18-AGA; 50 μM), and octanol (10 mM) (Figure 2A and B). The IP₃ levels in the presence of the gap junction blockers CBX, 18-AGA, and octanol were 2.29 ± 1.44 , 2.5 ± 0.3 , and $3.85 \pm 1.33 \times 10^{-13}$ moles, respectively. It has been reported that elevation of intracellular Ca^{2+} can also interfere with hemichannel opening (De Vuyst et al. 2006). BAPTA is a fast calcium-chelating reagent. Preincubation with BAPTA-AM to buffer intracellular Ca^{2+} significantly reduced the IP₃ release (Figure 2C). However, incubation with the P2x receptor blocker pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS; 50 μM) did not eliminate the IP₃ release (Figure 2C). The IP₃ release was still increased to $11.82 \pm 1.13 \times 10^{-13}$ moles by the reduction of extracellular Ca^{2+} in the presence of 50 μM PPADS; there was no significant decrease ($p=0.52$, t test) in comparison with the IP₃ release at 0 mM Ca^{2+} (Figure 2B). Verapamil is a selective P-glycoprotein inhibitor and can inhibit the adenosine triphosphate (ATP)-binding cassette (ABC) transporters. Application of 10

μM verapamil also did not eliminate IP_3 release (Figure 2C). The IP_3 release at 0 mM Ca^{2+} was $11.57 \pm 0.66 \times 10^{-13}$ moles in the presence of 10 μM verapamil. This further supports that IP_3 is released by hemichannels.

Membrane Tension Induced IP_3 Release

Mechanical stimulation can induce membrane tension to interfere with the hemichannel opening (Leybaert et al. 2003; Bao et al. 2004). Under mechanical stimulation, the IP_3 release was increased to $11.2 \pm 1.1 \times 10^{-13}$ moles (Figure 3). In this experiment, we also applied a hypotonic extracellular solution to induce cell membrane stress. The IP_3 release was increased from 3.3 ± 1.3 to $11.3 \pm 2.7 \times 10^{-13}$ moles as the osmolarity of the incubating solution was reduced from 300 to 275 mOsm (Figure 3). This incremental release was also eliminated by CBX. After application of 0.1 mM CBX, the IP_3 release was reduced to $1.44 \pm 0.11 \times 10^{-13}$ moles (Figure 3).

Hemichannel Dye Uptake in the Cochlear Sensory Epithelium

Hemichannel activity can be demonstrated by the dye-uptake assay. Figure 4 shows hemichannel dye uptake in the cochlear sensory epithelium. As previously reported (Zhao 2005a), the cochlear sensory epithelium had LY dye uptake under the low-bivalent condition (Figure 4A and B). Also, elevation of extracellular Ca^{2+} could diminish this dye uptake; the epithelium had no dye uptake in the incubation in the 10 mM Ca^{2+} ECS (Figure 4C and D). Further examination in the dissociated cell preparation showed that the cochlear supporting cell had dye uptake, whereas the outer hair cell, which has no connexin expression, had no dye uptake in the same incubation (Figure 4E and F). Coincubation with CBX (0.1 mM) also eliminated the dye uptake in the cochlear supporting cells (Figure 4G and H). These experiments further demonstrated that hemichannels in the cochlear sensory epithelium can also influx small molecules.

IP_3 Receptor Expression and Extracellular IP_3 Evoked Elevation of Ca^{2+} in the Cochlear Sensory Epithelium

IP_3 activates IP_3 receptors (IP_3Rs) to stimulate Ca^{2+} release from the intracellular Ca^{2+} store and external Ca^{2+} influxing to induce intracellular Ca^{2+} elevation (Berridge 1993; Dellis et al. 2006). Immunofluorescence staining showed that IP_3Rs are extensively expressed in the cochlear sensory epithelium (Figure 5). The intense labeling for IP_3Rs is visible in the cytoplasm (Figure 5A) and on the cochlear cell surface (Figure 5C to F). We further found that the extracellular application of IP_3 could elevate the intracellular Ca^{2+} concentration in the cochlear sensory epithelium (Figure 6). Ca^{2+} fluorescence images show that the cellular Ca^{2+} in the epithelium was elevated by perfusion of extracellular IP_3 (Figure 6A). After washout of IP_3 , the Ca^{2+} fluorescence was diminished. This IP_3 -evoked Ca^{2+} elevation was reversible and repeatable (Figure 6B), and could also be eliminated by gap junction hemichannel blockers (Figure 6C). Treatment with 0.1 mM CBX abolished the Ca^{2+} elevation (Figure 6C).

DISCUSSION

In this study, we used a fluorescence polarization technique to directly measure IP₃ concentration for assaying hemichannel IP₃ release in the cochlear sensory epithelium (Figures 2 and 3). We found that hemichannels in the cochlear sensory epithelium could release IP₃. The IP₃ release was increased from the nanomolar to micromolar levels by the reduction of extracellular Ca²⁺ and mechanical stress. We also found that the application of extracellular IP₃ elevated cellular Ca²⁺ in the cochlear sensory epithelium (Figure 6), indicating that IP₃ can act as an extracellular mediator to perform intercellular communication.

It has been well documented that hemichannels can function on the cell surface to release small cell-signaling molecules such as ATP and glutamate (Bennett et al. 2003; Goodenough and Paul 2003). This hemichannel-mediated ATP release can play an important role in intercellular signaling in the brain and retina (Kamermans et al. 2001; Thompson et al. 2006). In the cochlea, we have also reported that hemichannels can function in the cochlear sensory epithelium to release ATP to modify outer hair cell electromotility and regulate hearing function (Zhao 2005a; Zhao et al. 2005). In this experiment, we have found that hemichannels in the cochlear sensory epithelium can also release IP₃ (Figures 2 and 3). This result not only provides direct evidence that gap junction channels in the cochlea are permeable to IP₃, but also provides the first evidence that hemichannels can release IP₃ as well.

In this study, we have further demonstrated that extracellular application of IP₃ can elevate intracellular Ca²⁺ concentration (Figure 6), indicating that IP₃ can act as an extracellular mediator to participate in intercellular communication (Figure 7). IP₃ can mediate the adrenergic response and elevate intracellular Ca²⁺ concentration by activation of IP₃ receptors (Berridge 1993). Recently, it has been found that IP₃ can activate only a few (2~5) IP₃ receptors on the cell surface to dominant intracellular Ca²⁺ concentration (Dellis et al. 2006). IP₃ receptors are expressed extensively in the cochlear sensory epithelium (Figure 5). As shown by hemichannel dye uptake (Figure 4; also see Zhao 2005a), the extracellular IP₃ may reenter into the neighboring cells via hemichannel uptake and subsequently binds to IP₃ receptors to elevate intracellular Ca²⁺ concentration (Figures 6 and 7).

This hemichannel-mediated intercellular signaling may be able to achieve broad, long-distance intercellular communication and plays an important role in the IP₃-mediated intercellular signaling, given that IP₃ is rapidly degraded by enzymes in the cytoplasm. The lifetime of IP₃ in most cells is less than a few seconds (e.g., $t_{1/2}$ =4 s in liver) (Berg et al. 2001). So, it is difficult for IP₃ to travel a long distance in the cytoplasm via intracellular gap junction channels between cells. However, IP₃ in the extracellular space can survive long time because there are no specific enzymes to degrade it quickly. Indeed, we found that the IP₃ could be stable in the incubation solution up to 1 h and had no significant degradation (data not shown). Thus, IP₃ may be able to travel a long distance in the extracellular space to achieve broad, long-distance intercellular signaling (Figure 7).

Connexin mutations account for 70% to 80% of nonsyndromic deafness in children (Kelsell et al. 1997; Zhao et al. 2006). Although gap junctions play important roles in hearing, their roles in cochlear function are poorly understood. In the cochlea, the supporting cells employ gap junctions extensively. In contrast, the auditory sensory hair cells do not have gap junctions or connexin expressions (Kikuchi et al. 1995; Lautermann et al. 1998; Forge et al. 2003; Zhao and Yu 2006). Previously we proposed that the hair cells, which have no direct blood supply, receive nutrient and energy supplies from the supporting cells by means of mediators released through hemichannels (Zhao 2005b; Zhao et al. 2005). Our present study further supports this concept. Hemichannels in the cochlea can release ATP and IP₃, which can elevate intracellular Ca²⁺ to trigger and regulate a wide range of cellular processes, including cell fertilization (Berridge 1993). By extension, these data may also have implications for hearing loss related to connexin mutations and the possible mechanism by which these mutations cause hair cell dysfunction (Zhao et al. 2006). Mutations in these proteins may be likely to interrupt this crucial cochlear signaling function as well as the possible nutrient supply to the hair cells.

Recently, it has been found that pannexin as a new gap junction gene family can also encode gap junction proteins in vertebrates (Baranova et al. 2004; Shestopalov and Panchin 2008). Pannexin proteins can also form functional hemichannels on the cell surface to release ATP. Like connexin hemichannels, pannexin hemichannels can be gated by elevation of intracellular Ca²⁺ and mechanical stress, and are sensitive to CBX inhibition but insensitive to extracellular Ca²⁺ (Shestopalov and Panchin 2008). Our preliminary data show that pannexin has expression in the cochlear sensory epithelium (unpublished observation in our laboratory). IP₃ may also be able to be released via pannexin hemichannels. However, the measured IP₃ release was significantly reduced in 2 mM extracellular Ca²⁺ (Figures 2 and 3), indicating that pannexin hemichannels may not be a major contributor to this IP₃ release in the cochlear sensory epithelium. However, it cannot be completely ruled out that pannexin hemichannels can act as a partner to coparticipate in IP₃ release, just as they can release ATP, so as to play an important role in intercellular signaling.

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Figure 1.

The standard curve of IP₃ concentration measured by fluorescence polarization. The polarized signal (mP) is inversely proportional to the amount of the IP₃. The curve represents fitting of the data to the Hill equation.

Figure 2.

Hemichannel-mediated IP₃ release in the cochlear sensory epithelium. (A, B) IP₃ release by the reduction of extracellular Ca²⁺ and blockage by gap junction channel blockers. The trace along the x-axis of A shows the procedure of cochlea incubating in different Ca²⁺ concentrations. The right y-axis indicates the concentration of IP₃ released normalized to the cochlear fluid volume (10 μl). (C) The IP₃ release under treatment with the P2x receptor blocker PPADS (50 μM), the ABC transporter inhibitor verapamil (Verap; 10 μM), and preincubation with the intracellular calcium chelating reagent BAPTA.

Figure 3.

IP₃ release in the cochlear sensory epithelium by osmolarity stress and mechanical stimulation. The cochlea was preincubated in normal extracellular solution (300 mOsm) and then switched to be incubated in a hypotonic extracellular solution (275 mOsm). The IP₃ release was increased by the hypotonic challenge and blocked by cotreatment with 0.1 mM CBX.

Figure 4.

Hemichannel dye uptake assay in the cochlear sensory epithelium. (A, B) LY dye uptake in the cochlear sensory epithelium under low-bivalent condition. A is a phase-contrast image of the epithelium. B is its fluorescence image, which had dye uptake after incubation with LY. (C, D) The epithelial dye uptake was eliminated by elevation of extracellular Ca^{2+} . The incubation was performed at 10 mM extracellular Ca^{2+} concentration. (E, F) Dye uptake in dissociated cochlear cells. The supporting pillar cell had dye uptake, but the outer hair cell, which has no connexin expression, did not have dye uptake in the same incubation (indicated by arrows). (G, H) Gap junction blocker blocks dye uptake in the cochlear supporting cell. An isolated cochlear supporting Hensen cell had no dye uptake in coincubation with 100 μM CBX. Scale bars=100 μm (A to D); 10 μm (E to H).

Figure 5.

Expression of IP₃ receptors (IP₃Rs) in the cochlear sensory epithelium. (A, B) Immunofluorescence labeling for IP₃R in the cochlear sensory epithelium. (C to F) Expression of IP₃Rs in the cochlear-supporting cells. Labeling is visible on the cell surface, in the cytosol, and on the nuclear membrane. Asterisks in E indicate lipid bubbles in Hensen cells that have no IP₃R labeling. Scale bars=50 μm (A, B); 20 μm (C, D); 10 μm (E, F).

Figure 6. Extracellular IP₃ evoked intracellular Ca²⁺ elevation in the cochlear sensory epithelium. (A) Time-lapse recording of Ca²⁺ fluorescence images in the cochlear sensory epithelium evoked by extracellular perfusion of 0.1 μ M IP₃. Scale bar=20 μ m. (B) Ca²⁺ fluorescence intensity in the cochlear sensory epithelium was continuously recorded. Horizontal bars represent extracellular perfusion of 0.1 μ M IP₃. (C) Blockage of Ca²⁺ elevation by coapplication of CBX. CBX eliminated the extracellular IP₃-triggered elevation in intracellular Ca²⁺ concentration. The sensory epithelium was coincubated with 0.1 mM CBX. Note a small increase at the beginning of the IP₃ perfusion induced by perfusion-induced mechanical disturbance.

Figure 7. Schematic drawing of hemichannel-mediated intercellular signaling pathway in the cochlea. IP₃ can pass through not only intact gap-junctional channels but also hemichannels to participate in intercellular signaling.