Published in final edited form as:

Hear Res. 2007 November ; 233(1-2): 40–45. doi:10.1016/j.heares.2007.07.005.

Mitochondria-activated cisternae generate the cell specific vesicles in auditory hair cells

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Abstract

A dense population of vesicles largely fills the infranuclear compartment of gerbil inner hair cells (IHCs). Although the nature of the cargo in these vesicles has not been determined, the absence of a Golgi apparatus from the IHC's basal compartment suggests that the vesicles lack the glycosylated protein that Golgi cisternae would provide. Instead, they likely possess neurotransmitter and function as synaptic vesicles. The morphologic mechanism for generating the vesicles also remains unexplained. Ultrastructural examination revealed a few discrete clusters of mitochondria in the IHC's basal compartment. The clustered mitochondria made contact either with intermingling single cisternae or with one end of an unique set of polarized parallel cisternae. Both of these cisternal forms belong to a novel, mitochondria-activated category of cisternae which transforms into aligned segments where contacting mitochondria. Mitochondria-activated cisternae also envelope the vesicles in Hensen bodies of outer hair cells (OHCs). Coexistence of the mitochondria-activated cisternae with a specialized population of cytoplasmic vesicles in both IHCs and OHCs implicated this type of cisterna in synthesis of the cell specific vesicles. Assumedly, the mitochondria-activated cisternae possess an ATPase of the Class IV type. This class of enzymes, also designated flippases, translocates aminophospholipid from the outer to inner leaflet of the lipid bilayer and appears thereby to induce a lipid asymmetry which leads to cisternal segmentation and then vesiculation. In support of such an interpretation, RT-PCR analysis demonstrated the presence of Class IV ATPase in the Organ of Corti.

Keywords

Vesiculogenesis; Hearing; Cochlea; Granular reticulum; ATPase; Mitochondria

1. Introduction

The cochlear inner hair cell (IHC) contains a dense population of cytoplasmic vesicles in the cell's infranuclear compartment (Spicer et al., 1999). Outer hair cell (OHC) profiles, on the other hand, express Hensen bodies that consist of vesicles collected into a discrete ring or sphere in the upper region of the cell. The cytologic and molecular entities mediating biogenesis of these specialized vesicle populations have not been ascertained.

Ultrastructural examination of the basal compartment of gerbil IHCs revealed an enigmatic structure composed of a set of several parallel cisternae of granular reticulum (Spicer et al., 1999). Re-evaluation of hair cells in the gerbil cochlea by electron microscopy was undertaken in the present study in search of further information concerning the full length morphology and apparent function of the sets of parallel cisternae. The results disclose structural polarization of this organelle and reveal its content of a newly recognized cisternal category which undergoes segmentation when contacted by mitochondria.

2. Materials and methods

Inner ears from eight 3–6-month-old Mongolian gerbils (*Meriones unguiculatis*) raised in a low-noise environment were used in this study. Young adult animals from this colony have consistently shown normal hearing, (Schmiedt, 1989; Mills et al., 1990). Inner ears also were collected from four 3-month-old Sprague–Dawley rats for RT-PCR analysis as described below. The animal use protocol was approved by the Animal Use and Care Committee of the Medical University of South Carolina under National Institutes of Health grant R01 DC00713.

2.1. Morphologic procedures

The gerbils were anesthetized with intraperitoneal urethane (1.5 g/kg) and were sacrificed by transcardial perfusion first with 10 ml of normal saline containing 0.1% NaNO₂ and then with 30 ml of a mixture of 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. After removing the stapes, opening the oval window and perforating the round window, 1.0 ml of fixative was perfused gently into the scala vestibuli through the oval window. The inner ears were dissected free and immersed in fixative overnight at 4 °C. The scalae were flushed with buffer, and then a 120 mM solution of ethylenediamine tetraacetic acid (EDTA), pH 7.0, was injected gently into the oval window. Decalcification was completed by immersion in 400 ml of EDTA with gentle stirring at room temperature for 2–3 days and with daily changes of the EDTA solution.

The cochleas were then flushed again with buffer prior to perfusion with a warm solution of 10% gelatin (300 bloom). They were chilled on ice, allowing the gelatin to solidify, and then bisected under a dissecting microscope. The half cochleas were rinsed (three times for 5 min each) with warm phosphate buffer (40 °C) to remove residual gelatin.

Postfixation was carried out with a solution prepared by slowly adding potassium ferrocyanide powder to 1% osmium tetroxide in distilled H₂O to reach a 1.5% concentration of the ferrocyanide (Rostgaard and Møller, 1980). This solution resembled that employed for glycogen demonstration (DeBrujin, 1968) and membrane preservation (Karnovsky, 1971) and has proven useful for visualizing canalicular reticulum (Spicer et al., 1999). The half cochleas were immersed for 2 h in this solution in the dark. After the staining reactions, the half cochleas were sliced into individual half turns, dehydrated, and embedded in LX112 epoxy resin.

Plastic sections approximately 1 µm thick were cut and stained with toluidine blue. Sections were examined to ascertain orientation of embedment and, where necessary, to reposition the block in the microtome for obtaining a radial plane of sectioning. Adjacent ultra thin sections were stained with uranyl acetate and lead citrate and were viewed with a JEOL-1210 transmission electron microscope. Digital images were scanned from photographic negatives by using Adobe Photoshop software (Adobe Systems, Mountain View, CA) and were not modified in any form.

2.2. RT-PCR analysis

Rat cochleas were chosen as a source of mRNA for type IV ATPase because coding sequence for gerbils is unavailable. Two male and two female adult Sprague-Dawley rats around 3 months of age were used in this study. The rats were anesthetized by intraperitoneal injection of urethane (1.5 g/kg body weight) and exsanguinated by transcardial perfusion with sterilized PBS. The inner ears were rapidly excised, the optic capsule was removed and the organ of Corti containing IHCs, OHCs and adjacent supporting epithelial cells was microdissected from all eight ears. 1 µg of total RNA isolated from this pooled sample was reverse transcribed into cDNA using a RETROscript® Kit (Ambion, Inc., Austin, TX). Primers for PCR were designed based on the conserved sequences from *Bos taurus* (GeneBank accession # U51100), mouse (GeneBank accession # U75321), and human (GeneBank accession # AF067 820). Primers used were as follows: set 1, P1S (sense, 5'-AATGGTGCATGGGAAATTGT-3') and P1AS (antisense, 5'-TTCTGCATCAGCTTGGTGTGTC-3'); set 2, P2S (sense, 5'-ATCGAGATCTGGTTTGCCTT-3') and P2AS (antisense, 5'-CAATGAACCCAGAAAACCTTGG-3'). The specimens were pre-heated at 95°C for 3 min followed by 35 cycles at 95 °C for 45 s, 55 °C for 1 min and 72 °C for 1.5 min. PCR products were separated on 1.5% agarose gels and the 419 bp and 221 bp PCR products were eluted and sequenced.

3. Results

The infranuclear compartment of IHCs enclosed fairly numerous mitochondria. These organelles were not distributed randomly in the cytosol, but instead lay collected into clusters located basolateral to the nucleus (Fig. 1). Although appearing as a few isolated groups of mitochondria in the thin sections, the mitochondria clusters presumably would comprise a ring of numerous clusters arranged intermittently around the nucleus in a three dimensional reconstruction. The clustered mitochondria established two newly observed forms of association with linear cisternae. In one form, single cisternae appeared colocated with the clusters, since such cisternae were only observed intermingled in mitochondrial clusters. These cisternae contacted the clustered mitochondria extensively, wrapping around and squeezing between them (Fig. 1). The cisternae invariably transformed into aligned dense walled segments at the sites of contact with mitochondria.

The other form of mitochondrial association with cisternae involved a polarized structure composed of a set of parallel cisternae (Fig. 2) (Spicer et al., 1999). The number of cisternal profiles varied, ranging up to six per set, apparently in accord with the plane of section. Ovoid blebs with flocculent luminal content were closely grouped on the cisternae at the presumed upstream end of the set (Fig. 2). At the opposite pole, each cisterna protruded into a tight cluster of mitochondria. These parallel cisternae likewise terminated with transition into dense walled segments where contacting one or more of the clustered mitochondria (Fig. 2). In addition, a previously observed uniform population of vesicles (Spicer et al., 1999) occupied the infranuclear cytosol of the IHC (Fig. 2).

Outer hair cells expressed a different specialized system also composed of vesicles. These vesicles lay collected into a ring-shaped or spherical structure, which has been designated the Hensen body. Cisternae, showing disruption into aligned dense walled segments, where contacting mitochondria, comprised a limiting boundary at the periphery of the Hensen body (Fig. 3). This rim of mitochondria-activated cisternae closely encircled and apparently confined the dense collection of vesicles in Hensen bodies.

To investigate the possible presence of type IV ATPase in auditory sensory hair cells, RT-PCR reactions were performed using mRNA isolated from the microdissected rat organ of Corti. The dissected region consisted of both IHCs and OHCs and of supporting epithelial cells. As

shown in Fig. 4, PCR products of the predicted size for two highly specific sequences for type IV ATPase were identified. Sequencing of the 419 bp and 221 bp products revealed 99% and 100% identity, respectively, with the predicted sequence in rat (GeneBank accession # XM223390).

4. Discussion

The sets of parallel cisternae observed in the basal half of IHCs and illustrated in a schematic (Fig. 5) were shown to consist of a unique type of rough endoplasmic reticulum (Spicer et al., 1999). This presumably holds true also for the individual segmenting cisternae intermingling with clustered mitochondria (Fig. 2). Endowed with ribosomes, granular reticulum comprises the cytologic structure that functions to synthesize nascent membrane and generate cytoplasmic vesicles. The sets of parallel cisternae then provide the structure that qualifies for biogenesis of the vesicles populating the IHC basal cytosol.

The fact that the sets of parallel cisternae and the diffuse population of presumed synaptic vesicles coexist as unique constituents in the IHC's basal compartment further indicates a dynamic relationship between them. Polarized structural features of the sets of parallel cisternae in full length views of the organelle support this prospect. Bleb-like bodies adhering to the presumed accreting pole of the organelle apparently reveal the morphologic correlate of the biosynthetic activity underlying genesis and growth of the cisternae (Fig. 2). At the opposite downstream terminus the parallel cisternae protrude into mitochondrial clusters and at points of contact with mitochondria dismantle into segments.

The segmented cisternae can plausibly be viewed as an intermediate phase of transformation of cisternae into vesicles. Their contact with mitochondria implies that cisternae in IHC basal cytosol resorb ATP and accordingly possess an ATPase. Content of a type IV ATPase or flippase would provide a mechanism for translocating aminophospholipid across the bilayer and establishing a lipid asymmetry requisite to deformation of the membrane and genesis of vesicles. It is proposed then that the sets of parallel cisternae contain a flippase which utilizes ATP from adjacent mitochondria to translocate aminophospholipid in the lipid bilayer and induce deformation of the membrane and vesicle genesis.

The sets of segmenting parallel cisternae in IHC basal cytosol could turnover in the course of incorporation into an autophagosome and digestion by the liposomal enzymes. Although the apical IHC region reveals lipofuscin bodies that most likely represent a residue from autophagy of apical canalicular reticulum, the basal IHC compartment lacks such structures. Absence of lipofuscin bodies from IHC basal cytosol supports the interpretation that the sets of parallel cisternae do not turnover by digestion in an autophagosome.

Vesicles budding from granular reticulum in secretory epithelia generally transport cargo protein to Golgi cisternae for glycosylation prior to secretion. Since the IHC's basal cytosol is devoid of Golgi cisternae, the vesicles apparently produced by its distinctive sets of parallel cisternae must lack secretory protein destined for post-translational modification. From this absence of a protein cargo, their location throughout IHC basal cytosol and their frequent association with synapses, these structures can be interpreted as synaptic vesicles containing neurotransmitter.

The sets of parallel cisternae in the IHC basal compartment differ from other forms of granular reticulum in transforming into dense walled segments, where squeezing between or wrapping around clustered mitochondria. The invariable contact between mitochondria and cisternae in these sets suggests that the segmenting cisternae take up ATP from the mitochondria and that they utilize it through possessing an ATPase. It can be postulated moreover that action of their ATPase induces the segmentation characteristic of the sets of parallel cisternae since, this

morphologic change occurs exclusively and invariably at the precise point of contact between mitochondrion and cisterna (Fig. 2). The segmentation of these cisternae might be explained, if their ATPase belonged to the type IV ATPase or flippase, which by translocating aminophospholipid across the bilayer induced a lipid asymmetry favorable to membrane deformation.

Biological membranes are characterized by an asymmetric distribution of lipids across the bilayer. The outer monolayer of the plasma membrane contains mostly phosphatidylcholine and sphingomyelin (Bretscher, 1972a, 1973), whereas the inner membrane leaflet is enriched with phosphatidylethanolamine and phosphatidylserine (Bretscher, 1972b; Verkleij et al., 1973; Rothman and Lenard, 1977; Op den Kamp, 1979). This asymmetric lipid distribution is maintained by a type of ATPase which translocates aminophospholipids to the membrane's inner monolayer and has been referred to as a flippase (Zachowski et al., 1989; reviewed in Daleke and Lyles, 2000). These type IV ATPases were purified from chromaffin granules and erythrocytes and belong to a family also designated as P-type ATPases (Kuhlbrandt, 2004).

Flippases play a role in promoting membrane traffic. They mediate endocytosis, for example, by inducing a transbilayer lipid imbalance requisite to budding of endocytic vesicles from plasmalemma (Pomorski et al., 2003). That such phospholipid flipping induces membrane budding and vesiculation was shown by the perturbed endocytic activity in mutant yeast with deficient flippases (Pomorski et al., 2003). By translocating aminophospholipids from outer to inner membrane leaflet the flippase could generate a transbilayer lipid asymmetry and thereby induce deformation, segmentation and vesiculation of the linear cisternal membrane.

Golgi cisternae produce vesicles that transport cargo protein for secretion in most secretory cells. However, the basal cytosol of IHCs is devoid of Golgi cisternae. Absence of Golgi cisternae leads to the interpretation that vesicles in this site lack protein cargo and may contain neurotransmitter. The rough ER has generally been recognized as budding vesicles that are targeted to Golgi cisternae and is not believed to contribute to membrane traffic downstream from the Golgi complex. Granular reticulum in the basal IHC region, however, differs morphologically from that found elsewhere both in lacking associated Golgi zones and undergoing segmentation at sites of contact with mitochondria. Segmenting cisternae, from their presumed content of a flippase, offer a plausible source for vesicle biogenesis. Colocalization of an unique set of parallel segmenting cisternae with an unique population of cytoplasmic vesicles in basal cytosol of IHCs is consistent with the view that the sets of parallel cisternae function to generate the vesicles.

Although association of Hensen bodies with mitochondria in OHCs was noted by Engström and Ades (1973) and Spoendlin (1970), the relation of Hensen body vesicles to mitochondria and segmenting cisternae was not. Mitochondria-associated segmented cisternae observed here most likely serve the same purpose whether enveloping vesicles collected into the Hensen bodies of OHCs (Fig. 4) or occupying subnuclear cytosol in IHC's (Fig. 2). Inner and outer hair cells alike then revealed coexistence of a specialized population of cytoplasmic vesicles with mitochondria-activated cisternae, further suggesting a role for these cisternae in vesicle genesis.

Acknowledgments

This work was supported by Grant R01 DC00713 from the National Institute on Deafness and Other Communication Disorders and conducted, in part, in a facility renovated with support from Grant C06 RR014516 from the Extramural Research Facilities Program of the National Center for Research Resources, National Institutes of Health.

References

- Bretscher MS. Phosphatidyl-ethanolamine: differential labeling in intact cells and cell ghosts of human erythrocytes by a membrane-impermeable reagent. *J. Mol. Biol.* 1972a;71(3):523–528. [PubMed: 4648341]
- Bretscher MS. Assymetrical lipid bilayer structure for biological membranes. *Nature-New Biol* 1972b; 236(61):11–12. [PubMed: 4502419]
- Bretscher MS. Membrane structure: some general principles. *Science* 1973;181(100):622–629. [PubMed: 4724478]
- Daleke DL, Lyles JV. Identification and purification of aminophospholipid flippases. *Biochim. Biophys. Acta* 2000;1486:108–127. [PubMed: 10856717]
- DeBruijn, WC. A modified OsO₄ (double) fixation procedure which selectively contrast glycogen; Proceedings of the 4th European Regulatory Conference on Electron microscopy; 1968. p. 65
- Engström, H.; Ades, HW. The ultrastructure of the organ of Corti. In: Friedman, I., editor. *The Ultrastructure of Sensory Organs*. North Holland: Amsterdam; 1973. p. 83-151.
- Karnovsky, MJ. Use of ferrocyanide-reduced osmium tetroxide in electron microscopy; Proceedings of the 14th Annual Meeting of the American Society of Cell Biology; 1971. p. 146
- Kuhlbrandt K. Biology, structure and mechanism of P-type ATPase. *Nature Rev./Mol.Biol* 2004;5:283.
- Mills JH, Schmiedt RA, Kulish LF. Age-related changes in auditory potentials of Mongolian gerbil. *Hear. Res* 1990;46:201–210. [PubMed: 2394633]
- Op den Kamp JAF. Lipid asymmetry in membranes. *Annu. Rev. Biochem* 1979;48:47–71. [PubMed: 382989]
- Pomorski T, Lombardi R, Riezman H, Devaux PF, van Meer G, Holthuis JCM. Drs2p-related P-type ATPases Dnf1p and Dnf2p are required for phospholipid translocation across the yeast plasma membrane and serve a role in endocytosis. *Mol. Biol. Cell* 2003;14:1240–1254. [PubMed: 12631737]
- Rostgaard J, Møller K. Localization of Na⁺,K⁺-ATPase to the inside of the basolateral cell membranes of epithelial cells of proximal and distal tubules in rabbit kidney. *Cell Tissue Res* 1980;212:17–28. [PubMed: 6254659]
- Rothman JE, Lenard J. Membrane asymmetry. *Science* 1977;195(4280):743–753. [PubMed: 402030]
- Schmiedt RA. Spontaneous rates, thresholds, and tuning of auditory nerve fibers: comparison between cat and gerbil. *Hear. Res* 1989;42:23–26. [PubMed: 2584157]
- Spicer S, Thomopoulos G, Schulte B. Novel membranous structures in apical and basal compartments of inner hair cells. *J. Comp. Neurol* 1999;409:424–437. [PubMed: 10379828]
- Spoendlin, H. *Ultrastructure of the Peripheral Nervous System and Sense Organs: Atlas of Normal and Pathological Anatomy*. Stuttgart: Georg Thieme Verlag; 1970. p. 452
- Verkleij AJ, Zwaal RFA, Roelofsens B, Comfurius P, Kastelijin D, Van Deenen LMN. The asymmetric distribution of phospholipids in the human red cell membrane. A combined study using phospholipases and freeze-etch electron microscopy. *Biochim. Biophys. Acta* 1973;323:178–1934. [PubMed: 4356540]
- Zachowski A, Henry J-P, Devaux PF. Control of transmembrane lipid asymmetry in chromaffin granules by an ATP-dependent protein. *Nature* 1989;340:75. [PubMed: 2544808]

Fig. 1.

A: An inner hair cell shows two clusters of mitochondria (arrows) below and lateral to the nucleus. Bar = 2.0 μm . B: Individual linear cisternae located inferior to a Golgi zone (G) spread throughout an infranuclear cluster of mitochondria. The cisternae transform into aligned dense walled segments (arrows) at sites of mitochondrial contact and thus belong to the mitochondria-activated category of cisternae. Bar = 0.3 μm .

Fig. 2.

A: A polarized set of parallel cisternae unique to inner hair cells borders the basolateral plasmalemma. Bleb-like spheres (arrow) bind to two short cisternae slightly separated from the inferior end of five longer parallel cisternae. At their upper terminus the parallel cisternae ascend into a cluster of nine mitochondrial profiles. The upward extensions of the cisternae dismantle into continuously aligned segments where contacting the outer and squeezing between the inner mitochondrial surfaces (arrow-heads). The mitochondrial-cisternal complex lies in a bed composed of a widespread population of presumed synaptic vesicles (Spicer et al., 1999) and could mediate biogenesis of the vesicles. Bar = 0.25 μ m. B: Another polarized

set consists of 6 parallel cisternae. Bar = 0.5 μm . C: Blebs bind at the inferior end of a set of five parallel cisternae. Bar = 0.3 μm .

Fig. 3. Two Hensen bodies in an outer hair cell consist of vesicles densely aggregated into a ring or sphere. Aligned cisternal fragments of variable length delimit the periphery of the Hensen bodies. These and other nearby cisternae (arrows) segment at points of contact with mitochondria. Bar = 0.3 μ m.

Fig. 4. RT-PCR analysis of mRNA encoding type IV ATPase in the organ of Corti. A single band of the predicted size was amplified from each primer pair. The PCR product size is 419 bp for primer set 1 and 221 bp for primer set 2, respectively.

Fig. 5.

A schematic representation shows polarized parallel cisternae ascending from short separated segments bordered by blebs (arrow) to the basal edge of a cluster of mitochondria. Apparent extensions of the cisternae spread upward and transform into aligned dense walled segments (arrowheads) where contacting mitochondria.