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REGULAR ARTICLE

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Dye-coupling of melanocytes with endothelial cells and pericytes in the cochlea of gerbils

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Abstract Intercellular connections via gap junctions in the stria vascularis, which constitutes the lateral wall of the cochlear duct, were investigated by the Lucifer yellow microinjection method with the aid of a confocal laser microscope. The dye injected into an intermediate cell (melanocyte) diffused into capillary endothelial cells and pericytes as well as other intermediate cells, basal cells, and fibrocytes in the spiral ligament; whereas the dye injected into a marginal cell (epithelial cell) was confined to the injected cell. The observation of dye-coupling between intermediate cells and endothelial cells and pericytes makes likely the possibility that these cells work together to play a role in the specific function of the stria vascularis (i.e., production of the positive endocochlear potential and the endolymph) and adds endothelial cells and pericytes to the current "two-cell model" of the stria vascularis.

Key words Stria vascularis · Intermediate cell · Lucifer yellow · Microinjection · Gap junction · Gerbil

Introduction

It is generally accepted that the stria vascularis in the cochlea produces K^+-rich endolymph and the positive endocochlear potential of +80 mV (EP; for review, see Dallos 1996). In spite of the critical role of the EP in the normal function of cochlear hair cells, the cellular mechanism underlying the production of the EP has not been agreed upon (for review, see Wangemann and Schacht 1996). The stria vascularis consists of several types of cells: marginal cells (epithelial cells), intermediate cells, basal cells, capillary endothelial cells, and pericytes. Among these cell types, intermediate cells are melanocytes that have migrated from the neural crest (Hilding and Ginzberg 1977; Steel and Barkway 1989). The stria vascularis is unique in its anatomical structure, which is characterized by the presence of: (1) two layers of cells connected by tight junctions, one formed by marginal cells and the other by basal cells, both of which isolate the inside of the stria vascularis; and (2) intermediate cells and a dense capillary network located in the space between the two cell layers mentioned above.

Connections between cells brought about by gap junctions allow direct intercellular communication and play important roles when groups of cells function as a tissue and an organ. With regard to the inner ear function, the importance of intercellular communication has been suggested (Carlisle et al. 1990; Masuda et al. 1994). Histological and immunohistochemical studies of the stria vascularis have revealed gap junctions between adjacent intermediate cells, between adjacent basal cells, between intermediate cells and basal cells, and between basal cells and fibrocytes in the spiral ligament (Forge 1984; Kikuchi et al. 1995; Reale et al. 1975). On the basis of these morphological findings, gap junctions have been thought to play important physiological roles in the stria vascularis (Schulte and Steel 1994; Wangemann 1995; Wangemann and Schacht 1996). However, gap junctions between intermediate cells and capillary endothelial cells and pericytes have not been reported up until now. Intercellular connections mediated by gap junctions can be detected under physiological conditions by following the diffusion of dye from one cell to another (Stewart 1981).

The aim of this study is to present evidence for dye-coupling of intermediate cells with other types of cells, including capillary endothelial cells and pericytes, and to consider the physiological relevance of intercellular connections between intermediate cells and endothelial cells and pericytes.

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Materials and methods

Gerbils (*Meriones unguiculatus*), 6–12 weeks old, were deeply anesthetized with pentobarbital sodium (50 mg/kg i.p.) and cochleae were obtained. The care and use of animals used in this study were approved by the Kochi Medical School Animal Care and Use Committee. Tissue strips of the lateral wall of the cochlear duct (1–1.5 mm long), including the stria vascularis and the spiral ligament, were dissected from the first and second cochlear turns and kept cold (4 ºC) for up to 60 min in an incubating solution until dye injection. The incubating solution contained: NaCl 110 mmol/l, Na-aspartate 40 mmol/l, $CaCl_2$ 0.7 mmol/l, $MgCl_2$ 1 mmol/l, K_2HPO_4 1.6 mmol/l, KH_2PO_4 0.4 mmol/l and glucose 5 mmol/l (pH 7.4).

Lucifer yellow CH (lithium salt; Molecular Probes, Eugene, OR, USA) was injected into a cell in each tissue strip under visual control with the aid of a fluorescence microscope (BX50WI; Olympus, Tokyo, Japan) equipped with a water-immersion objective lens (×60), a differential interference-contrast system, and micromanipulators. Tissue strips were transferred to a bath filled with the air-saturated incubating solution and were held in place by silver wires. Intermediate cells could be identified easily because these cells have black pigment in the cytoplasm (Fig. 1B). Tissue strips were observed by fluorescence microscopy throughout dye injection. Lucifer yellow CH (3%, i.e., 66 mmol/l solution) was injected electrophoretically via a conventional intracellular microelectrode made from borosilicate glass capillaries. The outer diameter of microelectrodes measured by scanning electron microscopy was 0.15–0.25 µm, and the tip resistance of these electrodes filled with 3% lucifer yellow was 0.6–1.4 GΩ. An inward square-pulse current (20–40 nA) of 0.5 s was injected at a frequency of 1 Hz for 2–6 min using the circuitry of an amplifier (707; WPI, New Haven, Conn., USA) and a voltage pulse generator (SEN7103; Nihonkohden, Tokyo, Japan). The above-mentioned method for current injection resulted in a pulsatile outflow of the dye from the electrode tip. Accordingly, the location of the electrode tip could be estimated from the position of the pulsatile dye-outflow. Tissue strips obtained from trials with a stable electrode-tip position throughout dye injection were processed for further analysis.

The tip of microelectrodes were inserted into intermediate cells via the marginal cell layer. The cell that emitted fluorescence stronger than any other cell in a tissue preparation was regarded as the dye-injected cell. Within 20 s after the onset of dye-injection, the typical cell shape of a dye-injected intermediate or marginal cell (see Results), visualized by the fluorescence of the dye in the cell, was observed and the possibility that the dye might be injected into the extracellular space was excluded. When the fluorescence from a tissue part which had not fluoresced at 10–20 s after the onset of dye injection became stronger than that from the initially visualized cell, such a condition was regarded as a result of inadvertent displacement of the electrode tip and the tissue preparation was discarded. Dye injection was performed at room temperature (24–26 ºC) if not otherwise noted. In order to exclude the effect of temperature, dye injection was also performed at 37 ºC.

Within 1 min after dye injection, the tissue strips were transferred into pH-buffered 4% formaldehyde and chemically fixed for 60–120 min, and then rinsed with the incubating solution. A carbohydrazide group in lucifer yellow is covalently linked to surrounding biomolecules during aldehyde fixation (Stewart 1981). Within 60 min after fixation, the tissue kept in the incubating solution was examined. Tissue strips for sections (n=3) were dehydrated in a series of ethanol baths of increasing concentration and embedded in Spurr's resin, and cut perpendicularly to the apical surface of marginal cells at an interval of 2 µm to make 0.5-µm-thick sections. These sections were stained with 0.5% methylene blue/0.5% azur II for 4 min and transmission light images were recorded. The tissue staining mentioned above was performed *after* obtaining fluorescence images in order to avoid interference by the fluorescence of methylene blue.

Both whole-tissue preparations and sections were observed using a confocal laser microscope system (LSM410; Zeiss, Germany) including a microscope (Axiovert 135; Zeiss). Excitation was performed using an argon laser (488 nm), and fluorescence images (more than 515 nm) were obtained using a high-magnification objective lens (NA 1.3, ×100) and a photomultiplier. To obtain stereoscopic images, serial optically sectioned images of whole-tissue preparations were recorded at an interval of 0.4 µm.

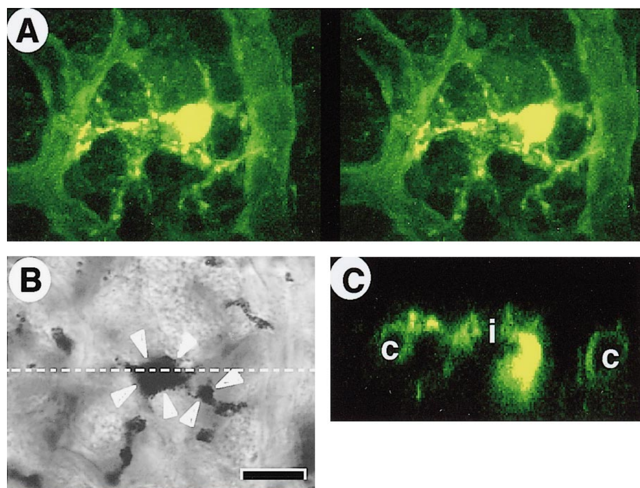


Fig. 1A–C Coupling of a dye-injected intermediate cell (melanocyte) with capillary-constituting cells. Representative images of ten similar tissue strips are shown. **A** Stereo pair of confocal laser micrographs. **B** Transmission light micrograph of the same visual field as in **A** focused on the cell body of the dye-injected intermediate cell. *Arrowheads* indicate black pigment in the dye-injected cell. *Interrupted line* indicates the sectioning plane for **C**. **C** Reconstructed *x-z* image. The apical surface of the tissue is directed upward. *Bar* 10 µm (*i* intermediate cell, *c* capillary)

Results

Intermediate cells could be identified by morphological characteristics, i.e., black pigment in the cytoplasm and dendrite-like projections (Figs. 1, 3, 4). Dye-injected marginal cells were easily distinguished from intermediate cells on the basis of the morphological characteristics of marginal cells, i.e., the flat hexagonal apical surface, the cylindrical shape, and the absence of pigment inclusions in the cell (Fig. 2). Lucifer yellow injected into a marginal cell at room temperature was confined to the injected cell, and no dye-coupling with other cells was observed (n=20; Fig. 2). In consideration of the possibility that gap junctions in marginal cells were affected by temperature, we also injected Lucifer yellow into a marginal cell at 37 ºC; no dye-coupling with other cells was observed (n=20).

As shown in Fig. 1, Lucifer yellow injected into an intermediate cell diffused into the cells that constitute capillaries (n=10). The dye-injected cell possessed typical morphological characteristics of the intermediate cell such as intracellular pigment and dendrite-like projections. Several projections of the dye-injected intermediate cell made contact with the capillary-constituting cells, and it is most likely that Lucifer yellow diffused into capillary-constituting cells via these projections. Intracellular spaces occupied by pigment were free of dye, probably because Lucifer yellow does not diffuse into pigment inclusions. Figure 3 shows a network constituted of dye-

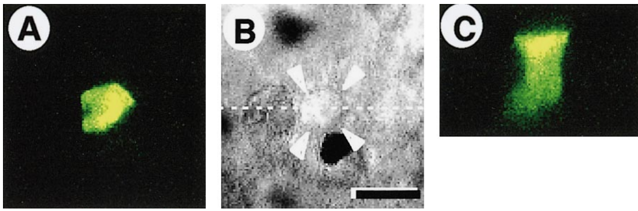


Fig. 2A–C Dye-injection into a marginal cell. Representative images of 20 similar tissue strips are shown. **A** Confocal laser micrograph, showing an optical section at approximately 1 µm from the apical surface of the marginal cell layer. **B** Transmission light micrograph of the same visual field as in **A** focused on the apical surface of the marginal cell layer. *Arrowheads* indicate approximate margin of the dye-injected cell. *Dotted line* indicates the sectioning plane for **C**. *Bar* 10 µm. **C** Reconstructed *x-z* image. The apical surface of the tissue is directed upward

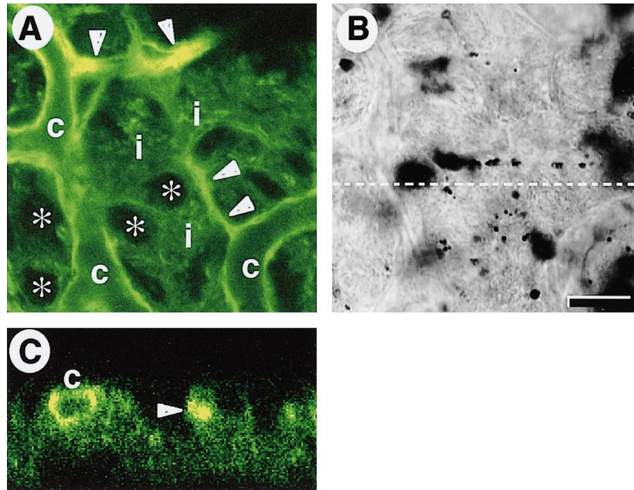
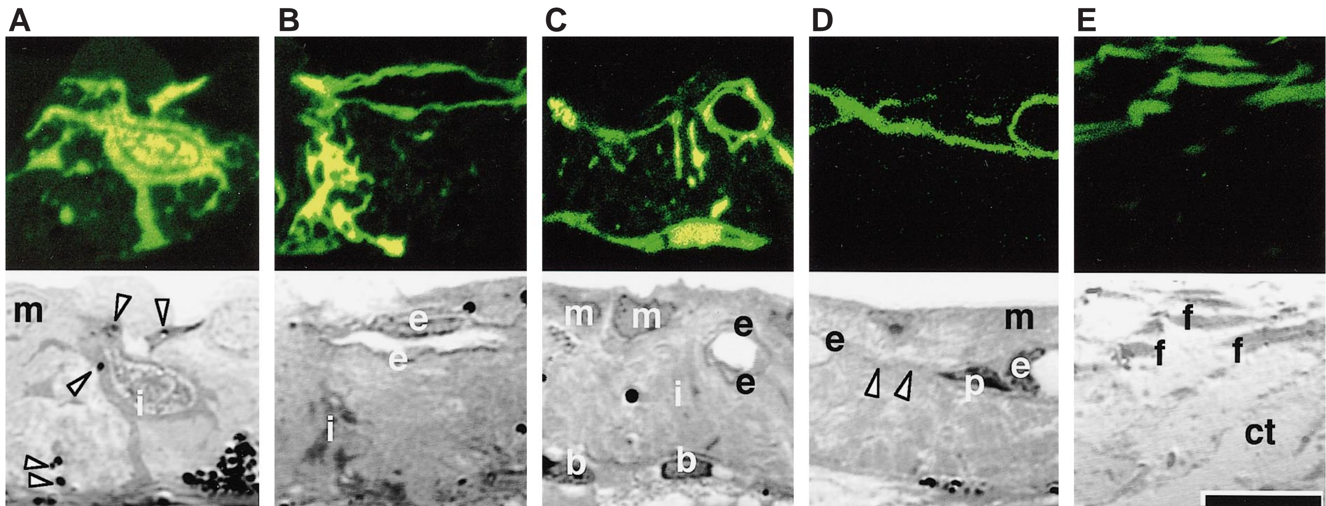


Fig. 3A–C Network formed by dye-coupled intermediate cells (melanocytes) and capillary constituting cells. Representative images of ten similar tissue strips. The dye-injected intermediate cell is out of the visual field. **A** Confocal laser micrograph, showing an optical section at approximately 5 µm from the apical surface of the marginal cell layer. *Asterisks* indicate spaces occupied by marginal cells. **B** Transmission light micrograph of the same visual field as in **A**. *Dotted line* indicates the sectioning plane for **C**. **C** Reconstructed *x-z* image. The apical surface of the tissue is directed upward. *Arrowheads* in **A** and **C**, projections without lumen. *Bar* 10 µm

coupled intermediate cells and capillary-constituting cells. As marginal cells were dye-coupled with neither intermediate cells nor capillary-constituting cells (Fig. 2), the spaces occupied by marginal cells looked like dye-free holes surrounded by intermediate cells (Fig. 3A, asterisks). Projections connected to capillary-constituting cells (Fig. 3A, arrowheads) were dye-coupled with intermediate cells and did not form a lumen (Fig. 3C, arrowhead).

Sections were examined to confirm dye-coupling of intermediate cells with endothelial cells and pericytes observed in the whole-tissue preparations and to investigate further the dye-coupling of intermediate cells with basal cells. As shown in Fig. 4, Lucifer yellow diffused from a dye-injected intermediate cell (Fig. 4A) which had a typical octopus-like shape and pigment inclusions. The apparent dye-coupling of an intermediate cell with endothelial cells was observed (Fig. 4B). Furthermore, Lucifer yellow diffused into basal cells (Fig. 4C), thus confirming the coupling of a basal cell with other basal cells and intermediate cells suggested by previous histological and immunohistochemical studies (Forge 1984; Kikuchi et al. 1995). In Fig. 4D, cytoplasm of a pericyte is stained as well as that of endothelial cells, and a projection from this pericyte (arrowheads) is dye-coupled with another endothelial cell. This type of pericyte might correspond to those cells with projections which are observed in whole-tissue preparations (Fig. 3). The connection between basal cells and fibrocytes in the spiral ligament, suggested by previous studies (Forge 1984; Kikuchi et al. 1995; Reale et al. 1975), was confirmed, since Lucifer

Fig. 4A–E Dye-coupling in the stria vascularis (**A–D**) and in the spiral ligament (**E**) observed in sections. Each panel consists of a fluorescence micrograph and a transmission light micrograph. Representative images of sections from three tissue strips. **A** Dye-injected intermediate cell. *Arrowheads* Pigment inclusions. **B** Coupling of an intermediate cell with endothelial cells. **C** Dye-positive endothelial cells, and basal cells. Dendrite-like processes of an intermediate cell are also seen. (**D**) Dye-positive endothelial cells and a pericyte. *Arrowheads* Projection of a pericyte to an adjacent capillary. (**E**) Dye-positive fibrocytes in the spiral ligament. *Bar* 10 µm (*m* apical part of marginal cell, *i* intermediate cell, *e* endothelial cell, *p* pericyte, *b* basal cell, *f* fibrocyte in the spiral ligament, *ct* connective tissue)



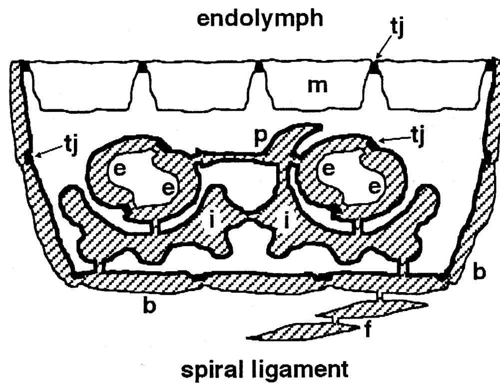


Fig. 5 Tissue model of the stria vascularis. *Hatched area* indicates dye-coupled cells observed in this study. *Thick solid lines* indicate functionally connected plasma membrane (*tj* tight junction)

yellow injected into an intermediate cell finally diffused into spindle-shaped cells in the spiral ligament (Fig. 4E).

Discussion

This study confirms the close association between the intermediate cell and strial capillaries (Conlee et al. 1989; Escobar et al. 1995; Hilding and Ginzberg 1977) and further presents evidence for dye-coupling between them. In Fig. 5, we propose an extended version of the "two-cell model" of the stria vascularis (Salt et al. 1987; Schulte and Steel 1994; Wangemann 1995), in which endothelial cells and pericytes are suggested to play an additional role. In general, dye-coupling between cells implies that molecules as large as Lucifer yellow CH (molecular weight 457) such as K^+, Na^+, Cl^-, glucose, and amino acids can move from the cytoplasm of a cell to that of coupled cells.

With regard to the physiological relevance of the dye-coupling of endothelial cells and pericytes with intermediate cells and basal cells, we first propose an electrophysiological function of endothelial cells and pericytes in cooperation with intermediate cells and basal cells in the production of the EP, since dye-coupled cells are electrically connected. This viewpoint agrees with the following reports: (1) the voltage step-up directly related to the generation of the EP is not likely to occur across the marginal cell layer (Wangemann 1995); (2) K^+-diffusion potential is closely related to the EP (Marcus et al. 1985; Takeuchi et al. 1996); and (3) the "two-cell model", which postulates the cooperation between marginal cells and other types of cells in the stria vascularis, is considered to be the most acceptable among several electrophysiological models of the stria vascularis (Wangemann and Schacht 1996). Assuming that K^+-diffusion potential is generated in the stria vascularis and that K^+ concentration in the intercellular space in the stria vascularis is low (Salt et al. 1987; Wangemann 1995; Wangemann and Schacht 1996), the plasma membrane shown by thick, solid lines in Fig. 5 is the most likely location for the voltage step-up resulting from K^+-diffusion. The large surface area of the plasma membrane, composed of intermediate cells, endothelial cells, pericytes, and basal cells may be indispensable for the production of the EP. The plasma membrane of endothelial cells facing the capillary lumen and that of the basal cells facing the spiral ligament are excluded from the membrane mentioned above (Fig. 5), since tight junctions join adjacent endothelial cells (Jahnke 1980; Sakagami et al. 1982) and also adjacent basal cells (Reale et al. 1975).

The second physiological role that can be put forward for the coupling of melanocytes with endothelial cells and pericytes may have to do with the metabolism of marginal cells, which secrete K^+-rich endolymph. Marginal cells are densely packed with mitochondria and display active aerobic respiration and a high energy-consumption rate (Marcus et al. 1978). Because of the close contact of intermediate cells with marginal cells (Conlee et al. 1989; Escobar et al. 1995; Hilding and Ginzberg 1977) and the absence of basal lamina around intermediate cells, it is likely that important metabolites such as glucose and amino acids are transported from the blood in strial capillaries to marginal cells via the cytoplasm of endothelial cells, pericytes, and intermediate cells connected by gap junctions. Similar metabolic coupling has been postulated for the coupling of endothelial cells with vascular smooth muscle cells (Fawcett 1994).

Other functions for the intermediate cells postulated until now include: (1) a role in normal development (Steel and Barkway 1989), and (2) the maintenance of homeostasis in this tissue (Mayer zum Gottesberge 1988). It is known that a congenital lack of intermediate cells results in severe interference in the generation of the EP (Cable et al. 1994; Kitamura et al. 1994; Schrott et al. 1990; Steel and Barkway 1989), suggesting a key role of intermediate cells in the normal function of the stria vascularis. The connection via gap junctions between fibrocytes in the spiral ligament and basal cells and that between basal cells and intermediate cells, both of which were confirmed in this study, have been regarded as pathways for the recycling of K^+ from the perilymph to the endolymph (Schulte and Steel 1994).

As endothelial cells and pericytes are usually covered by basal lamina, disruption of the basal lamina by melanocytes (Warfvinge et al. 1990) possibly underlies the formation of the dye-coupling routes. With regard to the connections between endothelial cells and other types of cells, those between endothelial cells and pericytes (Shepro and Morel 1993), and between endothelial cells and smooth muscle cells in the vascular wall (Fawcett 1994) have already been established.

Finally, connections of marginal cells via gap junctions with other marginal cells, intermediate cells, and basal cells were not identified in this study, though such connections have been suggested in morphological studies by electron microscopy (Forge 1984; Reale et al. 1975). There are at least two possibilities that explain the discrepancy between this study and previous studies. One is that particles in the plasma membrane such as Na^+-K^+-ATPase molecules (Carlisle et al. 1990; Mauns-

bach et al. 1980) might be interpreted as connexin molecules in the previous morphological studies. The other is that our experimental condition might shut down putative gap junctions in marginal cells.

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