

Intercellular communication in the supporting cells of the organ of Corti

Joseph Santos-Sacchi * and Peter Dallos

Auditory Physiology Laboratory (Audiology) and Department of Neurobiology and Physiology, Northwestern University, Evanston, Ill., U.S.A.

(Received 28 June 1982; accepted 25 October 1982)

We have directly tested the concept that the supporting cells of the organ of Corti are functionally coupled through gap junctions. *In vitro* and *in vivo* preparations were evaluated. Electrical measurements clearly show that the cells are coupled ionically. Voltage drops measured in neighboring cells in response to intracellular current injections indicate that current spread decays rapidly. Despite the existence of electrical coupling, fluorescent dye injection studies revealed no dye spread into adjacent cells, other than a few instances which were clearly artifactual. However, it is possible that dye spread is very slow and that dye in adjacent cells is diluted below visual detectability. In any case, dye coupling is remarkably poor compared to other electrically coupled tissues. The role of coupling in the supporting cells may be nutritive, considering the avascular nature of Corti's organ.

Key words: electrical coupling; dye coupling; organ of Corti.

Introduction

Over twenty years have passed since Furshpan and Potter [10] demonstrated that cytoplasmic communication can occur between individual cells. Subsequently, numerous studies have elucidated various details governing direct intercellular communication (for summaries see [17,22,14]). Indeed, in a variety of cell systems the existence of electrical as well as metabolic coupling has been established.

The cellular organelle thought to be responsible for such information transfer is the gap junction [11]. Its ultrastructure has been extensively studied by means of specific electronmicroscopic techniques, including freeze fracture and interstitial lanthanum infiltration [24,23,19]. However, despite the good correlation between intercellular coupling and the presence of gap junctions, the existence of gap junctional structures is not proof of electrical and/or metabolic coupling. For example, it is known that gap junctional structure is maintained following certain

* Present address and reprint requests: Department of Anatomy, Neurobiology Laboratory, Medical School, University of Puerto Rico, Old San Juan, PR 00901, U.S.A.

treatments which electrically uncoupled cells (e.g. glutaraldehyde, DNP, Li) ([25]; however, see [21]). Furthermore, Gulley and Reese [12], using freeze-fracture techniques, were able to find structures normally associated with gap junctions on the lateral walls of outer hair cells, which were exposed to the fluid filled spaces of Nuel. Clearly, as has been cautioned by Detwiler and Hodgkin [7], the electronmicroscopic demonstration of gap junctions is not sufficient to establish the existence or degree of intercellular coupling.

Several studies have indicated the presence of gap junctions in the adult mammalian inner ear [16,15,12]. An extensive array of gap junctions was demonstrated between all the supporting cells of the chinchilla and guinea pig organ of Corti. Freeze fracture revealed typical plaque-like structures and expansive areas of intramembranous particles. These results led to the conclusion that the supporting cells of the organ of Corti are electrically and metabolically coupled – a significant concept considering the metabolic demands and electrical activity of the organ. However, a direct evaluation of this proposal has never been attempted. We report here the results of experiments designed to evaluate such presumed intercellular coupling.

Materials and Methods

Animal preparations

In vivo. Young adult albino guinea pigs were used. This preparation has been previously described [5]. Briefly, after induction with urethane anesthesia, the animals were tracheotomized and the cochleas exposed through a ventrolateral approach. A fenestra was made in the bone over the stria vascularis in the third turn of the cochlea. Microelectrodes were inserted through the stria vascularis and the endolymphatic space into the supporting cells of the organ of Corti.

In vitro. Animals were decapitated after anesthetic induction and their cochleas removed. Two methods were employed to obtain specimens. Individual turns of the cochlea (stria vascularis removed) were microdissected out for study. Alternatively, only the bony capsule from the two apical most turns was removed. In this case the cochlea in toto, endolymphatic duct intact, was incubated in a microscope perfusion chamber. Electrode penetrations were made through Reissner's membrane into the supporting cells of the organ of Corti under visual control.

Electrophysiology

Electrodes were fabricated with a Brown–Flaming puller. Resistance of electrodes when filled with 3 M KCl ranged from 50 to 225 M Ω , with the mode around 120 M Ω . Lucifer Yellow, Procion Yellow, Niagara Sky Blue and 6'-carboxyfluorescein-filled electrodes were used for dye injection studies. Current pulses (–0.25 to –4 nA) were used to inject the fluorescent dyes.

For *in vivo* electrical coupling studies, double-barreled electrodes were made with tip-to-tip distances of 15–30 μ m. One electrode was bent according to the method of Hudspeth and Corey [13] so that the two electrode shanks could be placed parallel to

each other and epoxied together. This enabled us to penetrate adjacent Hensen's cells (diameter 15–25 μm) despite the inability to directly visualize the cells hidden beyond the stria vascularis. A stepping motor drive (Transvertex AB., Sweden) was used to advance the electrode assembly through the stria vascularis and into the organ of Corti's supporting cells.

For *in vitro* electrical coupling studies, separate micromanipulators were used to place the electrodes into neighboring supporting cells. Tissue was perfused with M199 culture medium (Gibco, Long Island, N.Y., U.S.A.) with modified Hank's salts (1.3 mg/100 ml Na_2HCO_3 , 10 mM Hepes, adjusted to pH 7.4 after equilibration with 5% CO_2 /95% O_2). The microscope perfusion chamber was maintained at $37 \pm 1^\circ\text{C}$. The cells were visualized and photographed with a Zeiss Standard microscope, equipped for epifluorescence.

Recordings and dye injections were made with either a commercial high-input impedance, capacitance-compensated amplifier (Mentor) or a dual-channel device constructed according to Fein [8] utilizing AD545 operational amplifiers (Analog Devices, Mass., U.S.A.). Both devices are capable of recording during current injection. Data were plotted on a strip chart recorder and in some instances electrical coupling was analyzed using a signal averager (Nicolet).

Cell input resistance was measured by injecting small negative currents (-0.25 to -1 nA) through the recording electrode and measuring the voltage drop across the cell membrane. Electrode resistance was linear in this current range. Current pulses from -1 to -10 nA were used to evaluate cell coupling. Coupling indices include coupling ratio (voltage drop in cell 2/voltage drop in cell 1, in response to current injected into cell 1; [1]) and mutual resistance (voltage drop in cell 2/current injected into cell 1; [7]).

Hensen's cells remained in good condition for up to several hours *in vitro*, as evidenced by interference optics and resting potentials. Experiments were completed before swelling and autolysis occurred, and they typically lasted from 1 to 2 h.

Light microscopy

For *in vivo* experiments, cochleas were fixed from 1 to 2 h after dye injection. Fixative was either 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, or 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. Desired portions of the cochlea were dissected out in buffer and either examined in surface mount or dehydrated in alcohols, embedded in Spurr and serially sectioned at about 12 μm . Injected cells were visualized with epifluorescence, using a BP450-490 nm exciting filter with a high-pressure mercury lamp. Photographs were taken with Kodak Ektachrome (ASA 200) and developed with the E-6 process (Unicolor). *In vitro* dye injection was monitored during experiments with brightfield and epifluorescence.

Results

In vivo

Upon passage through the stria vascularis in the *in vivo* preparation, the positive

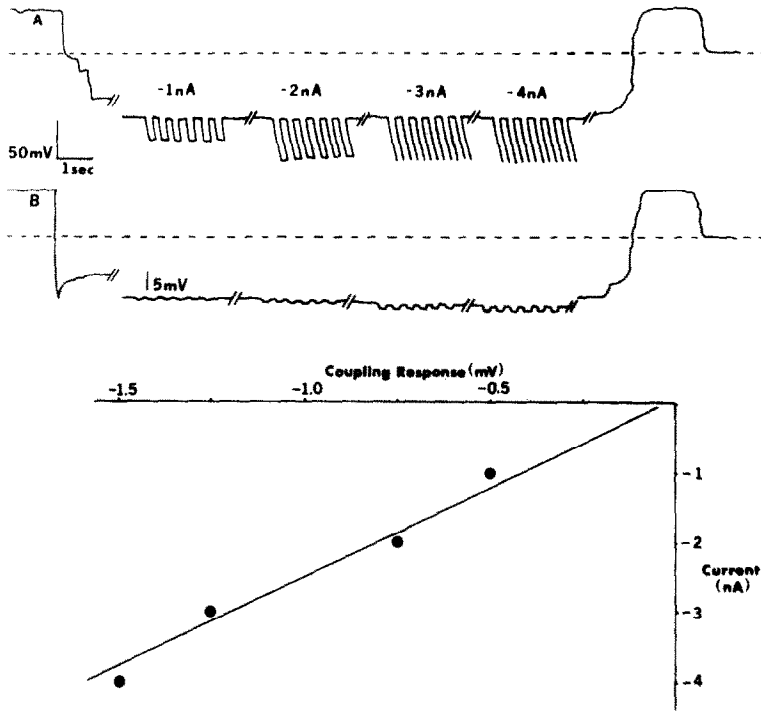


Fig. 1. Record of in vivo electrical coupling between adjacent Hensen's cells. Trace begins with electrode in endolymphatic space where the potential is near +70 mV. The sudden reversal of sign indicates that Hensen's cells are penetrated. Negative current pulses were injected in cell A at magnitudes of 1, 2, 3 and 4 nA. Bridge unbalancing in trace A indicates that cell A has an input resistance of 35 M Ω . Responses in cell B are linearly dependent upon current injection magnitude. Mutual resistance of these cells is 0.5 M Ω . The endolymphatic potential is again observed upon removal of electrodes from the cells, and after removal from the cochlear duct the potential drops to zero.

endocochlear potential is encountered (Fig. 1). After advancing the electrodes about 100–150 μ m further, the Hensen's cells are contacted. The stable membrane potentials averaged -70 ± 13 mV ($n = 87$). Input resistances are on the order of 35 ± 24 M Ω ($n = 37$). *

Fig. 1 illustrates the measurement of electrical coupling between adjacent Hensen's

* There is some evidence that the use of the single electrode technique to measure input resistance may overestimate the actual value by a factor of eight [27]. Apparently, the problem is that the resistance of the electrode must be taken into account in the calculations. Electrode resistance may increase preferentially inside the cell, thereby providing incorrect estimates of cell resistance. It would appear that if the electrode resistance does change preferentially inside the cell, the value of that change might be dependent upon the initial resistance of the electrode. A positive correlation between electrode resistance and Hensen's cell resistance might than be expected if the electrode were influencing the measurements. However, no such correlation was found in our measurements (Pearson $r = 0.013$).

cells. Membrane potentials of the cells are near -80 mV. Negative current pulses in cell A produce a voltage drop in that cell, proportional to its input resistance. Simultaneously, a voltage drop is noted in cell B. In this instance the voltage drop in cell B is considerably smaller than that in cell A, indicating a small but measurable coupling. The mutual resistance (see Materials and Methods) here is about 0.5 M Ω . Mutual resistances of up to about 1 M Ω have been found in the *in vivo* preparation. Mutual resistances with the current injecting electrode intracellular and the recording electrode immediately extracellular are typically less than 0.2 M Ω , indicating that spread is not through extracellular pathways. Fig. 1 also demonstrates that increasing the magnitude of the current injection produces a corresponding and proportional increase in cell B's response.

Despite the existence of electrical coupling, dye studies have failed to show spread into adjacent cells (Fig. 3a, b). Over 75 cells have been marked. Dye spread has occurred only on rare occasions when obvious mechanical trauma, i.e. from microdissecting prior to fixation, had been causal. Perhaps artifactual cytoplasmic bridges had formed between cells [4].

In vitro

An *in vitro* preparation was considered important in order to evaluate dye coupling fully. The direct visualization of dye injection under fluorescence microscopy eliminates the uncertainty associated with the processing required after *in vivo* injections. Initially, preparations for *in vitro* studies were obtained by microdissecting free the apical or third turn of the cochlea, after removing the stria vascularis. An

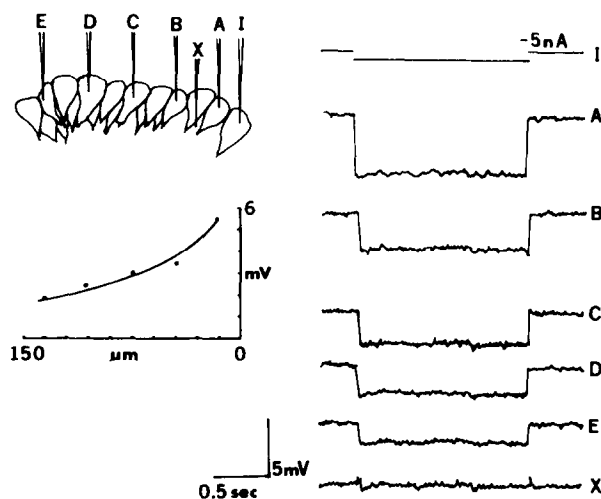


Fig. 2. Spatial analysis of electrical coupling in Hensen's cells *in vitro*. Current electrode *I* remained stationary while a roving electrode sampled intracellular responses to -5 nA current pulses at various inter-electrode distances. Responses were signal averaged. Response magnitudes indicate a fairly rapid decay as a function of inter-electrode distance, with a decay to one-third the adjacent cell response at about 130 μ m in this instance.

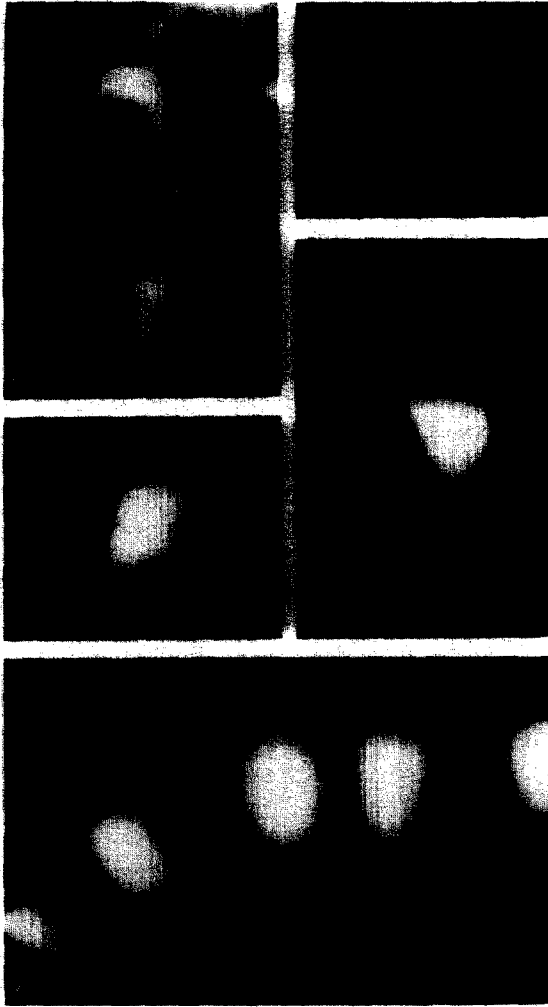


Fig. 3. (a) In vivo Procion Yellow injected outer pillar cell. Adjacent supporting cells above, below and in the plane of section were not stained. $800\times$. (b) In vivo Procion Yellow injected Hensen's cell. Note lipid vacuole is unstained. Adjacent cells demonstrate no detectable dye fluorescence. $470\times$. (c) In vitro Hensen's cells exhibiting Lucifer Yellow spread from a single injected cell. Five cells are stained. No spread was noted during dye injection (ca. 5 min). However, upon rebound of cells after difficult electrode removal, dye spread occurred within seconds. No further equilibration of dye occurred. $600\times$. (d) In vitro Lucifer Yellow injected Hensen's cell. Note lack of dye spread. This was typical of such experiments. Brightfield and epifluorescence. $600\times$. (e) In vitro preparation of Hensen's cells individually injected over a period of about 30 min. Each Lucifer Yellow injected cell is separated by an uninjected cell. $1000\times$.

analysis of these preparations revealed that cell potentials were depolarized as compared to in vivo preparations. Dye injections in these cells never demonstrated spread, except when physical trauma, e.g. difficult electrode removal, caused dye to enter adjacent cells (Fig. 3c).

By using a whole cochlea preparation, it is possible to maintain the functional integrity of the supporting cells of the inner ear. With endolymphatic duct intact, penetrations into Hensen's cells through Riessner's membrane permitted recordings of resting potentials (-63 ± 10 mV; $n = 111$) that were similar although statistically significantly different in magnitude to those measured in vivo (Student's *t*-test: $P < 0.001$). Electrical coupling was also similar to that in the in vivo preparation. Fig. 2 demonstrates coupling between Hensen's cells, as a function of inter-electrode distance. These data represent one of the better examples of spatial current spread. Current injecting electrode I remained stationary while the recording electrode sampled responses from cells at about 40 μm steps distal. The magnitude of the voltage drop in neighboring cells (Fig. 2A–E) in response to current injections (-5 nA, 1.5 s on–off) is dependent upon the distance from the current injected cell; the coupling response decays fairly rapidly as a function of increasing distance. The mutual resistance of the immediately adjacent coupled cells is about 1.0 $\text{M}\Omega$. Mutual resistances up to 2.0 $\text{M}\Omega$ have been found in this type of preparation. Note that the voltage drop in the extracellular space is miniscule (Fig. 2, X). Input resistance of these cells was also similar to that of cells in vivo.

Dye injections of Lucifer Yellow and 6'-carboxyfluorescein in over 75 cells demonstrated no dye spread into adjacent cells (Fig. 3d). The cells were observed for over an hour after injection. It was often possible to inject a row of single cells separated by uninjected cells (Fig. 3e).

Discussion

Electrical measurements clearly indicate that ionic coupling in the supporting cells of the organ of Corti is present both in vivo and in well maintained in vitro preparations. Coupling characteristics are similar for both preparations, showing mutual resistances around 1–2 $\text{M}\Omega$ for immediately adjacent Hensen's cells. Difficulty arises when attempts are made to determine coupling ratios because a single electrode was used to deliver current and measure voltage drops simultaneously. If the single electrode resistance measures were correct, this would mean that coupling ratios are poor. A comparison of mutual resistances of Hensen's cells and turtle retinal cones, for example [7], would seem to substantiate this contention. In these experiments, mutual resistances in better penetrations of neighboring cones averaged about 29 $\text{M}\Omega$ at a mean separation of 20 μm ; input resistance of cones were reported to be 24 $\text{M}\Omega$. Thus, turtle cones are much better coupled than Hensen's cells, if indeed measures of Hensen's cell input resistance are valid. *

* It is possible to obtain information on coupling ratios by comparing coupling responses in neighboring cells distal to the current electrode. That is, with a stationary current electrode and a roving measuring

Another indication of coupling efficiency is the analysis of spatial characteristics of intercellular current spread. At distances ranging from 2 to 9 Hensen's cell diameters ($\approx 45\text{--}130\ \mu\text{m}$) from the current injecting electrode, the coupling response is already about $1/3$ the response in the immediately adjacent cell. Sachs et al. [26] found a 50% reduction in coupling response magnitude from 3 to 5 cells distal in isolated gastric tubules. In contrast, Shueteze and Goodenough [29] demonstrated in the embryonic chick lens that the magnitude of the coupling response remained the same up to $80\ \mu\text{m}$ removed from the current injecting electrode. Furthermore, dye spread of intracellularly injected Lucifer Yellow is rapid and pervasive; it was reported that the whole chick lens can be stained given a sufficient length of time. Similar strong coupling has been found in other cell systems, e.g. insect gland epithelium and rat liver [18,20]. In rat liver, fluorescent dye spread occurs rapidly in over a dozen cells. This is in marked distinction from our dye studies.

About a decade ago it was thought that embryonic cells during certain developmental stages could maintain electrical coupling despite the inability to permit dye transfer [28,2,30]. A careful re-evaluation of this phenomenon has led Bennett et al. [4] to conclude that dye transfer does occur if cells are electrically coupled. The inability to observe dye spread, despite the presence of electrical coupling, in the supporting cells of the organ of Corti may represent a very slow passage of dye. However, it should be emphasized that observations were made up to several hours after dye injection. Yet it cannot be ruled out that such a slow spread would allow for dye dilution below visual detectability. Dye was observed in cells other than the injected ones only in those cases where supporting cells were physically traumatized, i.e. roughly manipulated. Spread in these instances was very rapid (less than 1 min) and may have been due to the artifactual formation of cytoplasmic bridges, although pathways through gap junctions cannot be dismissed. Nevertheless, dye coupling in the inner ear is clearly not at all like that in other well coupled cell systems, i.e. liver or lens.

Recently, Fraser and Bode [9] have shown that previous studies which demonstrated a lack of dye coupling in epithelial cells of *Hydra* [6] were due to dye injections into cellular vacuoles. It is important to note that this was not the case in our Hensen's cell studies. Although Hensen's cells in the apical and third turn of the guinea pig cochlea do contain large lipid vacuoles, cell penetrations always showed high negative resting potentials, and furthermore, after injection of dyes whole cells were filled but vacuoles were unstained.

The significance of electrical coupling in the supporting cells of the mammalian organ of Corti is obscure. Weiss et al. [31] have presented indirect evidence that hair

electrode, it has been found (e.g. Fig. 2) that voltage attenuation between Hensen's cells can be relatively low. Coupling ratios can be determined from this information and are found to average greater than 0.6. This indicates that input resistance of Hensen's cells may be *lower* than that measured directly with a single electrode. Attempts to measure input resistance with two electrodes have been difficult, but in a few presumably successful cases, measures have shown resistances below $10\ \text{M}\Omega$. In these cases adjacent cells have coupling ratios of about 0.5. Of course, it is possible that the penetration of cells the size of Hensen's cells with two electrodes may be injurious and reduce input resistance.

cells and supporting cells in the alligator lizard cochlea are electrically coupled. Thus, lateral hair cell interactions may be mediated through supporting cells in this case. However, in mammalia effects upon sensory transduction are apparently non-existent; no gap junctions are present between hair cells and supporting cells in the adult [16,15,12]. Furthermore, we have shown [5] that the magnitudes of receptor potentials of mammalian hair cells (inner and outer) are much larger than potentials recorded in any supporting cell type. Indeed, responses in intercellular spaces are also larger than in supporting cells, indicating that responses to sound in the latter are picked up from the extracellular space. The fact that spatial spread of current through supporting cells is not great further limits possible interactions.

A more likely role for intercellular communication in the organ is nutritive or metabolic. Such cooperation among the supporting cells may be requisite considering the avascular nature of Corti's organ proper. In fact, this may account for the continued survival of supporting cells under adverse conditions which destroy uncommunicating hair cells. A difficulty with this nutritive hypothesis is the consistent finding that dye spread is absent. The molecular weights of the fluorescent dyes used in this study are similar to those of many metabolites. Again, the inability to find dye spread may be due to very slow dye passage and dilution of dye below visual detectability. Further investigations are required to resolve this issue.

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