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Endolymphatic potassium of the chicken vestibule during embryonic development

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Abstract

The endolymph fills the lumen of the inner ear membranous labyrinth. Its ionic composition is unique in vertebrates as an extracellular fluid for its high-K^+/low-Na^+ concentration. The endolymph is actively secreted by specialized cells located in the vestibular and cochlear epithelia. We have investigated the early phases of endolymph secretion by measuring the endolymphatic K^+ concentration in the chicken vestibular system during pre-hatching development. Measurements were done by inserting K^+-selective microelectrodes in chicken embryo ampullae dissected at different developmental stages from embryonic day 9 up to embryonic day 21 (day of hatching). We found that the K^+ concentration is low (<10 mM/L) up to embryonic day 11, afterward it increases steeply to reach a plateau level of about 140 mM/L at embryonic day 19–21. We have developed a short-term in vitro model of endolymph secretion by culturing vestibular ampullae dissected from embryonic day 11 chicken embryos for a few days. The preparation reproduced a double compartment system where the luminal K^+ concentration increased along with the days of culturing. This model could be important for (1) investigating the development of cellular mechanisms contributing to endolymph homeostasis and (2) testing compounds that influence those mechanisms.

Keywords: Inner ear; Semicircular canal; Organotypic culture; Endolymph; Development

The inner ear houses the receptors for hearing and equilibrium; the hair cells. Acoustic and vestibular stimuli modulate the openings of mechano-sensitive cation channels located at the apical hair bundle (Hudspeth, 1989), through which Ca^{2+} and K^+ ions flow into the hair cell, thus producing a cell membrane depolarization (Omori, 1984). The resulting receptor potential is also shaped by voltage-dependent Ca and K channels localized at the basolateral membrane. Ca^{2+} inflow further depolarizes the cell and sustains transmitter release (Spassova et al., 2001), whereas K^+ outflow repolarizes the cell. Thus, K^+ ions are responsible for both hair cell depolarization, entering through the apical mechano-sensitive channels, and hair cell repolarization, flowing out through the basolateral voltage-dependent K channels. This is a consequence of the dramatic differences in ionic composition between the extracellular solutions bathing the apical or the basolateral region of the hair cells, which are called endolymph or perilymph, respectively. It has been known from the early 1950s (Smith et al., 1954) that endolymph is more similar to an intracellular than an extracellular fluid since the predominant cation is K^+ not Na^+. In contrast, perilymph is analogous to a normal extracellular fluid. Because of the ionic composition of these two fluids, the resulting chemo-electric force drives K^+ into the hair cell at the apical membrane, and out of the hair cell at the basolateral membrane.

The inner ear endolymph is almost unique as an extracellular medium. Some of the cellular mechanisms responsible for secretion of mammalian endolymph have been identified (reviewed in Ferrary and Sterkers, 1998). Endolymph production and homeostasis is a complex process that requires different types of membrane transporters, as well as different types of specialized cells. The cellular and sub-cellular organization appears during development at the time of onset of endolymph production. In mammals the rise of potassium concentration in the endolymphatic space occurs

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between the 4th and 8th day after birth (Anniko and Nordemar, 1980). In chickens, the endolymph is already formed before hatching (Runhaar et al., 1991), although it is not known when the endolymph starts to build up and what its progression is during development.

The present work was intended:

1) To describe the early phase of endolymph production in the developing chicken whose inner ear development has already been studied extensively.
2) To produce an in vitro model of endolymph secretion during development.

1. Experimental procedures

Experiments were carried out on chicken embryos. A total number of 177 chicken embryos at different developmental stages were used, subdivided as follows: 44 chicken embryos for single-barrel endolymphatic K^+ measurements (of these 34 provided useful data); 30 for endo-ampullary potential measurements (of these 23 provided useful data for the dissected ampullae and 5 for the cultured ampullae); 59 for double-barrel endolymphatic K^+ measurements (of these 43 provided useful data); 39 for organotypic cultures (of these 23 provide useful data for endolymphatic K^+ concentration); and finally 5 embryos were used to test for anesthesia. All procedures used were approved by the Ministero Italiano della Sanità and conformed to NIH guidelines.

Fertilized chicken eggs (domestic chicken, Cobb variety) were obtained from a local supplier and incubated at 38.3 °C in high humidity.

In a first set of experiments, we investigated embryos from embryonic day 9 (E9), the stage when the ampullae become large enough to insert a microelectrode to E21, which corresponds to hatching day. When staged according to the criteria of Hamburger and Hamilton (1951), we observed that the development of our embryos consistently lagged behind the standard atlas up to E19, most probably due to the higher incubation temperature used by Hamburger and Hamilton (39.4 °C) during the first nine days of incubation. The correspondence between our days of incubation (E) and the stages of development provided by Hamburger and Hamilton was as follows: E9 (35), E10 (36−), E12 (36+), E13 (38), E14 (39−), E15 (39+), E16 (40+), E17 (42), E18 (43+), E19 (44+), E20 (45), E21 (E46). For clarity, only the embryonic day (E) will be used throughout this paper.

Endolymphatic K^+ concentration was measured shortly after the animal was sacrificed. In vivo measurements in embryos younger than E15 proved impractical: anesthesia, induced by ketamine hydrochloride (Farmaceutici Gellini, Italy) injection under the skin of the chicken embryo back, could not be calibrated mainly because it was difficult to judge when the animal died during the experiment. Consequently, we chose to sacrifice the embryo and perform a rapid dissection; in this way we were able to record 5–10 min after death.

1.1. Surgical techniques

Two different surgical approaches were adopted, depending on the embryonic age: up to E15, the embryos were rapidly removed from the eggs, decapitated, the cranium was opened medially, the brain removed, and the portion of the skull with the two parietal bones containing the membranous labyrinths was isolated. This was pinned to a small piece of corkboard glued to the bottom of a home-made Perspex chamber (diameter 12 cm, height 2.5 cm) containing about 100 ml of warm (38 °C) normal extracellular physiological solution (composition: NaCl 145 mM, KCl 3 mM, $MgCl_2$ 0.6 mM, HEPES 15 mM, $CaCl_2$ 2 mM; pH 7.4). The liquid in the chamber was kept at 38 °C by means of a thermostat. Under oblique fiber optic illumination, a small hole was produced using a fine forceps in the cartilaginous tissue above the anterior ampulla, through which the single or the double-barrel microelectrode was introduced by means of a manual micromanipulator, and moved downward to penetrate the ampullary wall. The operation was visualized with the use of a stereo zoom (10–40×) microscope (Zeiss, Germany). Penetration inside the ampulla was detected by a sudden rise in the K^+-selective microelectrode potential. From E16, the ampullae became considerably larger, and could be observed and reached without dissecting the entire labyrinth. Therefore, after decapitation, only the temporal bone portion covering the anterior and lateral ampullae was removed. The head was then pinned to a small piece of corkboard glued to the bottom of a home-made Perspex chamber (diameter 12 cm, height 5 cm), filled with about 250 ml of warm normal extracellular physiological solution. No oxygenation of the recording chamber fluid or fluid perfusion were performed.

1.2. Organotypic culture

For organotypic cultures, anterior semicircular canals were dissected from E11 chicken embryos. We chose E11 ampullae, since the endolymphatic K^+ concentration started to increase significantly from E12. Therefore, if we were to see a significant increase of K^+ concentration in cultured ampullae, this would have occurred in vitro. No other ages were sampled.

The procedure was as follows: the egg shell was cleaned with alcohol, and dissection was carried out as above, but using a stereo zoom (10–63×) microscope (Nikon, Japan) under a sterile horizontal laminar flow hood in a dish containing sterile Hank's Balanced Salt Solution (HBSS, H9269, Sigma–Aldrich, MO, USA). Only a very short stump of the semicircular canal duct was left, by which we picked up the ampullae (see Fig. 1A). After dissection, each ampulla was rinsed with sterile Dulbecco's Modified Eagle's

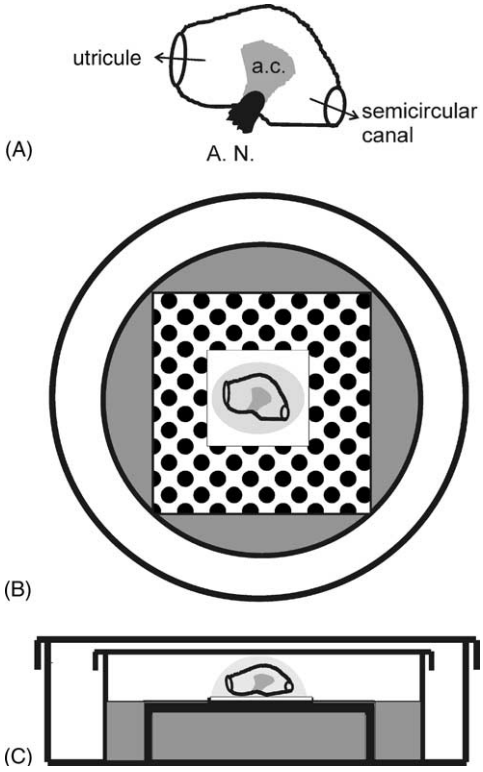


Fig. 1. Organotypic culture. (A) Schematic drawing of a vertical ampulla; AN, ampullary nerve; ac, ampullary crista. (B) Schematic drawing of the culture chamber; view from above. (C) Schematic drawing of the culture chamber; side view. The ampulla is shown inside a drop of Matrigel Basement Membrane Matrix (light grey), placed upon a membrane filter (white) positioned on a perforated stainless steel support made in the shape of a small table (black and white grid). The whole is in a small (35 mm × 10 mm) vented Petri dish, filled up to the membrane filter with DME tissue culture (dark grey). The Petri dish containing the DME is housed in a vented Petri dish of greater size (60 mm × 15 mm). This ensemble, constituting the culturing chamber was placed in the incubator.

Medium (DME, D6046, Sigma–Aldrich, MO, USA) and placed in a small drop of Matrigel Basement Membrane Matrix (40234A, Becton Dickinson Labware, MA, USA—Matrigel Basement Membrane Matrix has been reported to support growth, differentiation, and regeneration of several cell types, as referenced in the Product specification sheet). The Matrigel drops were pre-positioned over a strip of polycarbonate membrane filter (13040, Osmonics, CA, USA). The culturing procedure was adapted from Garrido et al. (1998). The inclusion of the ampulla in the gelatinous Matrix drop proved essential in preventing a flattening of the ampulla, and therefore an occlusion of the ampullary lumen, while allowing exchange for gases and nutrients. Each strip with the preparation was then positioned over a stainless steel support, which we had cut and shaped from a perforated stainless steel sheet (hole diameter 2 mm, S/S 430 RS Components Ltd, UK), and all was placed in a Petri dish. The described ensemble (Fig. 1B, C) constituted the culture chamber, which was filled to the level of the polycarbonate filter with sterile culture medium. This consisted of DME added with fetal bovine serum (v/v, 13%, F9665, Sigma–Aldrich, MO, USA), insulin (1 μg/ml, S9137, Sigma–Aldrich, MO, USA), penicillin (100 U.I./ml, P3032 Sigma–Aldrich, MO, USA), fungizone (1.4 μg/ml) or streptomycin (100 μM/ml, S9137, Sigma–Aldrich, MO, USA), L-ascorbic acid (0.3 mg/ml, A4403, Sigma–Aldrich), ferrous sulphate (0.45 μg/ml, F8633, Sigma–Aldrich, MO, USA), and hydrocortisone (1 μg/ml, Ho135, Sigma–Aldrich, MO, USA). The culture chambers were set in a sterile incubator and maintained at 37 °C in a 95%/5% O_2/CO_2 humidity saturated atmosphere. The culture medium was replaced every two days under sterile conditions.

In less then one day of culture, both the utricular and canal openings were closed and the ampullae took a spherical shape, so that an ampullary lumen was again separated from the external medium. For intra-ampullary measurement with the K^+-selective microelectrode, we replaced the culture medium with normal extracellular physiological solution. The entire culture chamber was positioned in the same recording chamber as above, properly adapted, and the microelectrode tip was simply advanced through the matrigel and the ampullary wall inside the ampullary lumen.

To check for survival of hair cells and inspect the overall architecture of the sensory epithelium in the cultured ampullae, a few of these were sliced by means of a vibroslicer, and the tissue slices were observed using an upright microscope (Zeiss Axioskop, Germany), and in a few cases, photographed (Canon A-1, Canon Inc., Japan). The technique used to slice the ampulla was previously reported in detail, along with pictures of slices of normal chicken embryo ampullae for comparison (Masetto et al., 2003). Briefly, the cultured ampulla, still in the matrigel drop, was included in a block of agar, which was immersed in partially frozen normal extracellular physiological solution, and cut with a vibrotome (Campden, UK) along the ampullary crista longitudinal axis. Slice thickness varied between 150 and 250 μm.

1.3. K^+ selective electrodes—double-barrel

Double-barrel glass capillaries with an inner filament (2GC150F-10—Clark Electromedical Instrument, UK) were pulled to a final tip diameter, for each barrel, of 10–20 μm. In this way the two tips, partially fused together, had a maximal diameter of 40 μm. In order to avoid electrical shunting between the solutions filling the two barrels, the bottom portion of one barrel was shortened by about 5 mm using a ceramic cutter. Both barrels were cleaned by blowing air inside, and the shorter one, representing the reference microlectrode, was filled with distilled water. The tip of the other capillary was filled with 5% (v/v) dichlorodimethyl-silane in carbon tetrachloride. The contents of both barrels were then evacuated, and the micropipettes were heated for 4 h at 200 °C. Afterwards, the short barrel was filled with 1 M NaCl solution, while the other barrel was filled with a 0.15 M KCl solution. Finally, the tip of the latter was filled

with the K^+ ion exchange resin (Fluka, potassium ionophore I—cocktail B 60398, Switzerland). Ag/AgCl wires were inserted in the micropipettes, and connected to a dual electrometer amplifier (WPI FD223, USA). Once the double-barrel microelectrode was inserted into the lumen of the ampulla, the potential read by the amplifier, which resulted from the difference potential arising at the tips of the two barrels, was a function of the endolymphatic K^+ concentration only. Following calibration of the microelectrodes as before, the potential read by the amplifier was correlated to the endolymphatic K^+ concentration. The main characteristics of the microelectrodes were: K^+-sensitive barrel = 730 MΩ (±81; $n = 59$); slope = 56 mV/decade (±2, $n = 59$); selectivity coefficient = log K_{KNa} = 3.5. Reference barrel: 900 KΩ (±110; $n = 59$).

1.4. K^+ selective electrodes—single-barrel

The technique used to make K^+-selective microelectrodes followed the one initially proposed by Neher and Lux (1973) and adapted by Valli et al. (1990). Glass capillaries with an inner filament (GC150F—Clark Electromedical Instrument, UK) were pulled to form micropipettes with tip diameters of 15–30 μm. Smaller tips broke during penetration of the ampullary wall. The last 300–400 μm of the micropipette tips were coated with 5% (v/v) dichlorodimethylsilane in carbon tetrachloride to make them hydrophobic, and heated for 4 h at 200 °C. The micropipettes were then filled with a 0.1 M KCl solution and their tips immersed in the same K^+ ion exchange resin as above which, by capillarity, filled the portion coated with silane. Ag/AgCl wires were inserted into the micropipettes, and the microelectrodes were connected to one input of the electrometer amplifier. The second input of the amplifier was connected to the reference electrode (an Ag/AgCl pellet immersed in the bath chamber). Every K^+-sensitive microelectrode was calibrated before and at the end of each experiment by measuring its voltage output in solutions with a known K^+ content (1, 10, 100, 150 mM; NaCl substituted for KCl to maintain an osmolarity of 318 m Osm; the calibrated solutions also contained $NaHCO_3$ 1.2 mM, NaH_2PO_4 0.17 mM, and $CaCl_2$ 1.8 mM; pH 7.4). If the calibration values differed by more than 10% before and after the experiment (which happened very rarely) results were not included in the analysis.

The main characteristics of K^+-sensitive microelectrodes may be summarized as follows: resistance: 460 MΩ (±52; $n = 83$); slope: 53 mV/decade (±1.9; $n = 83$); selectivity coefficient = log K_{KNa} = −3.5. We did not check for possible differences in K^+ endolymphatic concentration due to microelectrode tip position inside the ampullae.

1.5. Conventional microelectrodes for measuring the transepithelial voltage

To measure the transepithelial voltage in our preparation, we used conventional microelectrodes inserted into the ampulla. Glass capillaries (GC150F—Clark Electromedical Instrument, UK) were pulled to a tip diameter of 10–20 μm, filled with NaCl 1 M, and connected via an Ag/AgCl wire to the voltage amplifier. Pipette resistance was 850 KΩ (±85; $n = 30$).

1.6. Analysis

Single measurements lasting a few minutes were sampled digitally using an analog-to-digital converter coupled to a personal computer, analyzed and plotted on paper. When the intra-ampullary recording was prolonged for tens of minutes, responses were recorded on a chart recorder. Analyses of data were performed with Origin™ (Microcal Software Inc., USA). For statistical analysis, F-test and Student's t-test were performed with Excel™ (Microsoft Corporation, USA). Figures were prepared with CorelDraw™ (Corel Corporation). Throughout the text, the average results are expressed in the following format: mean (±standard deviation; n = number of cases). In figures, error bars indicate ±1 standard deviation unless otherwise specified.

2. Results

2.1. Endolymphatic K^+ concentration—double-barrel

Fig. 2A shows a representative trace for a double-barrel microelectrode recording. A rapid rise in voltage occurred when the microelectrode tip, following penetration of the ampullary wall, began to read the intra-ampullary K^+ concentration. The voltage read at the microelectrode tip remained constant throughout the impalement. This indicates that impalement did not result in a rapid outflow of endolymphatic K^+, possibly because the epithelium sealed around the glass pipettes. When the microelectrode was withdrawn from the ampulla, the voltage often failed to return immediately to the reference voltage value probably because the endolymph leaked out through the hole made by the microelectrode. As in the case of this recording, however, voltage (K^+ concentration) returned to reference (bath) value when the microelectrode tip was withdrawn further from the ampulla.

Results obtained with double-barrel microelectrodes are summarized in Fig. 2B (hollow squares). Endolymphatic K^+ concentration at E9–11 was less than 10 mM, but it increased rapidly to above 100 mM at later developmental stages, reaching a quasi-plateau level after E17.

Single values for K^+ concentration measured with the double-barrel microelectrodes were (in mM): 5, 5.5, 5, 5 (E9); 9 (E10); 8, 15 (E11); 37, 23, 32, 42 (E12); 77, 47, 44, 40, 42 (E13); 70, 53, 94, 98, 98 (E14); 110, 115 (E15); 102, 141, 102 (E16); 86, 135, 135, 119, 110 (E17); 115, 135 (E18); 153, 147, 115 (E19); 105, 152, 106, 159 (E20); 134, 135, 135 (E21); $n = 43$.

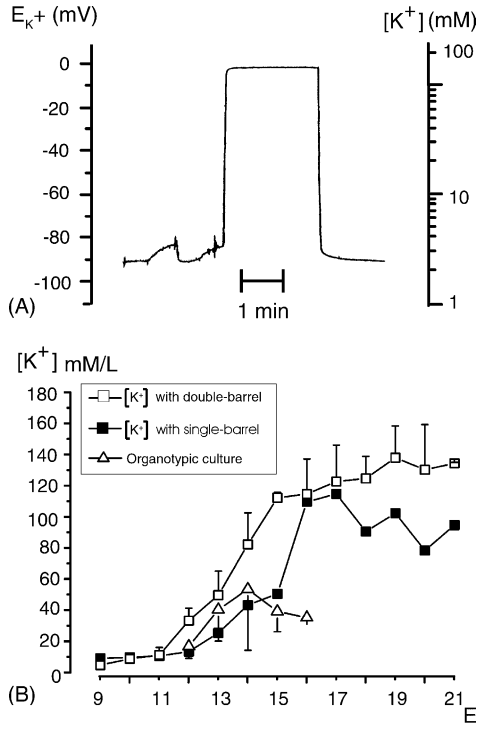


Fig. 2. Endolymphatic K^+ concentration. (A) Time course of double-barrel microelectrode measurement before, during, and following penetration in one ampulla (representative trace for an E16 chicken embryo). The trace depicts the time course of microelectrode reading before, during, and following penetration in the ampulla. The two vertical scales indicate the actual voltage read by the differential amplifier (left), and the K^+ ions concentration (right) calculated on the basis of prior microelectrode calibration (see Section 1). Horizontal scale bar = 1 min. Note that a first attempt to penetrate the ampullary wall failed, whereas at the second attempt the initially slow depolarization, likely reflecting the microelectrode tip pushing upon the ampullary wall, was followed by a rapid rise once the tip penetrated inside the ampulla. (B) Diagram showing mean values (±standard deviation) for endo-ampullary K^+ concentration. Squares refer to measurements performed on freshly dissected ampullae (hollow squares: double-barrel microelectrodes; filled squares: single-barrel microelectrodes), while triangles refer to measurements performed on cultured ampullae with single-barrel microelectrodes. In the abscissa, E refers to the chicken embryonic day at which the ampullae were dissected (squares), or to the corresponding day of development in ovo for cultured ampullae (triangles). Note that, for better clarity, standard deviations for single-barrel measurements in dissected ampullae (same data as Fig. 3B) have been omitted here, while only the plus or minus standard deviations are shown for double-barrel or organotypic data, respectively.

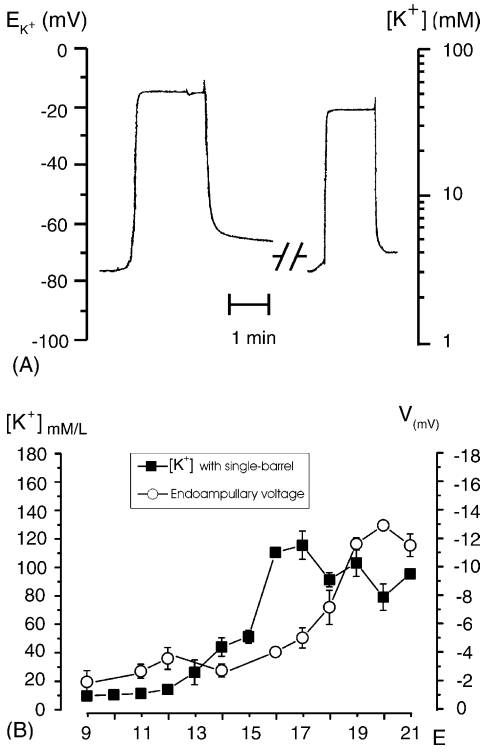


Fig. 3. Endolymphatic K^+ concentration—single-barrel microelectrodes. (A) Representative traces showing two measurements obtained from the two contra-lateral anterior ampullae at two different time intervals following animal death (see text). Each trace depicts the time course of microelectrode reading before, during, and following penetration in the ampulla. Recordings refer to an E15 chicken embryo. The two vertical scales are as in Fig. 2A. Horizontal scale bar = 1 min. (B) Diagram showing mean values (±standard deviation) for endo-ampullary K^+ concentration measured as above (filled squared), and the endo-ampullary voltage measured with conventional microelectrodes (circles), both as a function of embryonic day (E).

2.2. *Endolymphatic K^+ concentration—single-barrel*

Recordings with the double-barrel electrodes were sometimes difficult, particularly at early developmental stages, because the microelectrode tip pushed significantly against the ampullary wall before penetrating to the lumen. This could have produced endolymph leakage from the lumen. Therefore, we also recorded the endolymphatic K^+ concentration with single-barrel microelectrodes. Measurements with single-barrel microelectrodes were relatively easy: the single tip smoothly penetrated through the ampullary wall even in younger embryos, whose ampullae are very small (at E12 the ampulla diameter at the crista level is about 0.7 mm) and delicate. A representative recording at E15 is shown in Fig. 3A. The two traces refer to microelectrode measurements 15 and 35 min after animal death, respectively, in the two contra-lateral (left and right) anterior ampullae from the same chicken embryo. The lower value found in the second (contra-lateral) ampulla is most likely due to K^+ gradient dissipation in time (20 min passed between the two measurements – see Discussion), since both measurements were made with the same microelectrode in the same animal, and showed a similar time course and reliability (i.e. the measured value was stable throughout the recording period).

As shown in Fig. 3B (filled squares), from E9 to E11 the K^+ concentration in the endolymph was steadily around 10 mM, and it started to rise steeply from E12. At E16–17 the K^+ concentration reached a peak, and then slightly declined.

When comparing these results with those obtained with double-barrel microelectrodes, it clearly appears that the data overlap up to E11 (see Fig. 2B for a direct comparison).

Mean values for endolymphatic K^+ concentration at E9–11 were in fact 10 mM/L (±1.7; $n = 5$) with single-barrel microelectrodes versus 7.4 ± 3.7 mM/L with double-barrel microelectrodes; statistical analysis indicated that variances (F-test) and means (Student's t-test) were not significantly different ($P = 0.2$). From E12 the endolymphatic K^+ concentration increased steeply, and the values measured with single-barrel microelectrodes were generally smaller than those measured with double-barrel microelectrodes. The maximal value found was 122 mM (versus 159 mM with the double-barrel microelectrodes). Single values for K^+ concentration measured in each ampulla were (in mM): 11, 7, 10 (E9); 10 (E10); 11, 10.8 (E11); 12, 13, 16 (E12); 35, 18, 24 (E13); 37, 50, 43.5 (E14); 53, 48, 56, 46 (E15); 110 (E16); 108, 122 (E17); 86, 96, 90.5 (E18); 103, 93, 112 (E19); 94, 70, 78, 73, 79 (E20); 95 (E21); $n = 34$.

The results obtained by the single-barrel technique could have been contaminated if a transepithelial voltage was present across the ampullary wall. While a large transepithelial voltage across the cochlea is known to exist (several tens of mV), in the vestibule this is expected to be only a few mV (Ferrary and Sterkers, 1998). Nonetheless, because of the logarithmic relationship between the K^+ concentration and the voltage read, at high endolymphatic K^+ concentration, even small differences in mV would result in large errors in the estimated K^+ concentration. We therefore checked for the presence of a significant transepithelial voltage in our preparations.

2.3. Transepithelial voltage

To test for the presence of an endo-ampullary potential relative to the bath, we used conventional microelectrodes (see Section 1). In fact, a transepithelial voltage was present, which was negative inside the ampulla, and whose amplitude significantly increased with embryo age. The recorded value of the transepithelial voltage was quite constant for ten of minutes (not shown). Results are summarized in Fig. 3B (circles). Thus, the K^+ concentration measured with single-barrel microelectrodes was actually underestimated, the degree of underestimation increasing with embryos' age. In fact, since the voltage read by the amplifier becomes progressively less negative by increasing the endolymphatic K^+ concentration, if a negative contaminating potential (here the endo-ampullary voltage) adds to the voltage read by the microelectrode, the actual K^+ concentration in the ampulla is underestimated. Single values for the endo-ampullary voltage were (in mV): −2.2, −1, −2.5 (E9); −3, −2.3 (E11); −3, −4.1 (E12); −2.6, −3.2, −2.3 (E14); −4 (E16); −5.6, −4, −5.4, −5 (E17); −8, −6.3 (E18); −11.9, −11,3 (E19); −12.9 (E20); −11.2, −12.4, 10.9 (E21); $n = 23$.

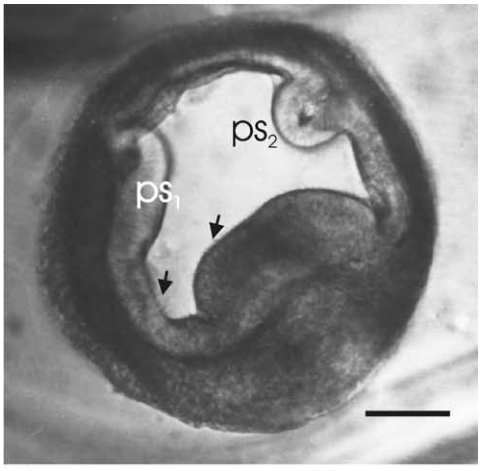
These results suggest that measurements performed with the double-barrel microelectrodes in the youngest embryos, despite their larger tips, were at least as reliable as those with single-barrel microelectrodes. Moreover, the decrease in endolymphatic K^+ observed after E17 by using single-barrel microelectrode was likely an artifact, i.e. it was due to a relatively larger underestimate of K^+ concentration after E17.

2.4. Intra-ampullary K^+ concentration in cultured ampullae

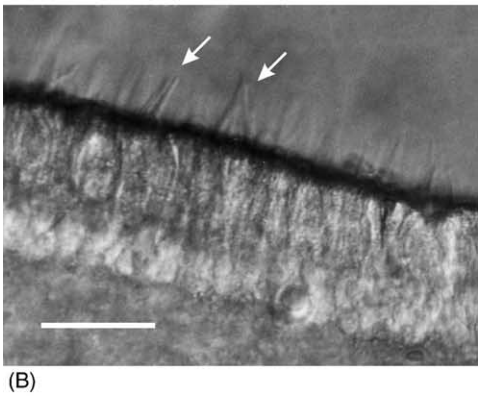
In a collateral set of experiments the ampullae were excised from E11 chicken embryos and cultured as described in Section 1. Following separation of the ampullae from the utricule and the semicircular canal ducts, the original endolymph was lost. The organ however showed a remarkable ability to seal the lesion during the day following dissection. Thus, an ampullary lumen was recreated in vitro, which could be visualized for microelectrode measurements by manipulating the position of the illuminating optic fiber. Moreover, the lumen, together with the overall architecture of the cultured ampullae, could be well appreciated by obtaining fresh tissue slices of the cultured ampullae. Fig. 4A shows, for example, a photomicrograph of a slice of an anterior ampulla grown in vitro for two days. Numerous short stereocilia could be seen protruding inside the lumen, indicating that hair cells were surviving (see, e.g. Fig. 4B). Moreover, a normal *planum semilunatum* (ps_1) was clearly distinguishable curving at one end of the sensory crista, while the second *planum semilunatum* (ps_2) was abnormal in shape, perhaps as a consequence of damage during the dissection (for a comparison with a slice from a normal vertical ampulla see Masetto et al., 2000). It can be presumed that normal tissue organization can be preserved with the organotypic culture here described, provided that damage to the ampullae during dissection is minimized.

The endolymphatic K^+ concentration in the cultured ampullae was initially measured with double-barrel microelectrodes. These measurements however appeared not reliable: the rise in potential was slow, and the ampullary wall appeared severely squashed by the microelectrode penetration. Therefore, data obtained with double-barrel microelectrodes were discarded. In a few cultured ampullae ("corresponding" to E12, $n = 2$; E14, $n = 1$; and E16, $n = 2$) we measured the transepithelial voltage by conventional microelectrodes, and we found it to be always ≤ -2 mV. Thus, we measured the endolymphatic K^+ concentration with single-barrel microelectrodes. A representative trace for an E11 chicken embryo ampulla cultured one day ("corresponding" to an E12) is shown in Fig. 4C. By assuming −2 mV as the value for the endo-ampullary voltage, and on the basis of the calibration curve for the single-barrel microelectrodes used, we estimated a maximal error of 13 mM in the ampulla showing the higher K^+ concentration (i.e. 86 mM). Errors could only be estimated because endo-ampullary voltage and endo-ampullary K^+ concentration were necessarily measured in different ampullae.

Average values for intra-ampullary K^+ concentration measured with single-barrel microelectrode are shown in Fig. 2B for direct comparison with previous results.



(A)



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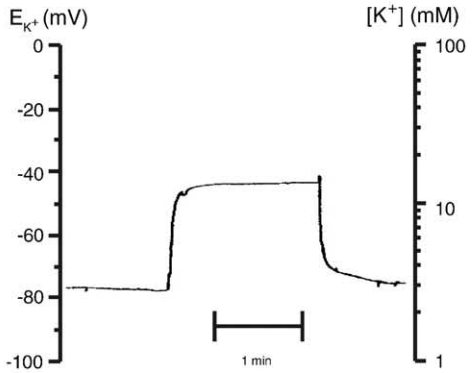

(C)

Fig. 4. Cultured ampulla. (A) Photomicrograph of a fresh tissue slice of a vertical ampulla dissected from an embryonic day 11 (E11) chicken embryo and cultured for two days (‘‘corresponding’’ to an E13). The sensory crista was cut along its longitudinal axes, so that both *plana semilunata* (ps_1 and ps_2) appear sectioned in the slice. Arrows point at the sensory epithelium where short stereocilia, barely visible, protrude in the lumen. Calibration bar = 100 μm. (B) Photomicrograph of a portion of the crista slice, obtained from an ampulla dissected at E11 cultured for two days (E13). Arrows point at sterocilia which are best in focus. The shape of a few hair cells can be distinguished. Calibration bar = 20 μm. (C) Time course of microelectrode measurement before, during, and following penetration in the ampulla (representative trace for an E11 chicken embryo ampulla cultured one day, i.e. ‘‘corresponding’’ to an E12). The two vertical scales indicate the actual voltage read by the differential amplifier (left) and the K^+ ions concentration (right) calculated on the basis of prior microelectrode calibration (see Section 1). Horizontal scale bar = 1 min.

Single values for K^+ concentration measured in each cultured ampulla with single-barrel microelectrodes were (in mM, not corrected for putative endo-ampullary voltage): 16, 14, 21, 30, 7, 10, 22, 13 (E12); 42, 61, 56, 34, 10 (E13); 65, 86, 10, 42 (E14); 57, 29, 30, 42 (E15); 37, 34 (E16); $n = 23$.

We found a clear trend for intraluminal K^+ concentration increase in vitro, which resembled that observed in the dissected ampullae up to E15 (Fig. 2B). Afterwards, the ampullae showed a progressive flattening, resulting in a reduction of the lumen volume, which after one week of culture almost disappeared. This impaired microelectrode measurements. It is possible that the progressive decrease of matrigel consistency we observed was responsible for the collapsing of the ampullae.

3. Discussion

In the present work we measured the endolymphatic K^+ concentration in the chicken embryo ampullae at different developmental stages. Measurements were performed with double- and single-barrel K^+-selective microelectrodes. Data obtained by the two techniques almost overlapped in the youngest embryos, and were also consistent in the older ones provided that corrections for the transepithelial voltage were taken into account. We believe, therefore, that results obtained with double-barrel microelectrodes are reliable in spite of the presumably large lesion produced at the ampullary wall. On the other hand, single-barrel microelectrodes allowed us to measure the endo-ampullary K^+ concentration in cultured ampullae.

A first point deserving discussion is that the present measurements were obtained after the animal was sacrificed (within 15 min post-mortem). Thus, the question arises of how close the values reported here are to those in the living animal. Unfortunately, no pertinent data for the embryo or adult chicken are available in literature. Moreover, a significant variability is found in literature for endolymphatic K^+ concentration values in living (anaesthetized) animals even for the same organ and animal species, due to different techniques of measurement, anaesthesia, animal respiration state, temperature, etc. By restricting the literature to the ion-selective microelectrode technique, in the anaesthetized chicken embryo, the cochlear endolymph K^+ concentration was 110 mM at E14, the earliest stage previously investigated, and reached an average value of 161 mM at E16 and older (Runhaar et al., 1991). However, the endolymphatic K^+ concentration is presumably lower in the chicken vestibule than in the cochlea, as reported for other animals. For example, in the pigeon the endolymph K^+ concentration was 133 mM in the ampulla versus 155 mM in the cochlea (Ninoyu et al., 1987). In the guinea pig the endolymph K^+ concentration was 130 mM in the ampulla versus 167 mM in the cochlea (Mori et al., 1987). The post-mortem K^+ concentration measured here, with an average value at E19–21 of 134.1 mM (±19.6; $n = 10$) is well in the

range of the expected value, when considering that the closest reference would be the adult pigeon vestibule, i.e. 133 mM (Ninoyu et al., 1987). Nonetheless, values found here might be slightly underestimated. It is presumable, in fact, that a slow K^+ gradient dissipation begins shortly after animal death. In the guinea pig cochlea, as soon as 2.5 min after the arrest of ventilation, a decline of endolymphatic K^+ concentration was already appreciable, although 60 min of anoxia was required to observe a 20% decrease (Melichar and Syka, 1977). Consistently, in one experiment on an E15 chicken embryo, where we prolonged the measurement, we read 56, 44, and 39 mM at 20, 40, and 80 min after the animal sacrifice (see also, e.g. Fig. 3A).

A related result is the negative endo-ampullary voltage found here, compared to the positive luminal voltage expected in vivo (Ferrary and Sterkers, 1998). This also, is a likely consequence of the animal's death. Consistent with this hypothesis, it was observed that during induced anoxia, the transepithelial voltage across the cochlea reversed to a negative value both in birds (Schmidt and Fernandez, 1962) and mammals (Konishi et al., 1961; Bosher, 1979). The same result was obtained by perfusion with ouabain, which blocks the Na–K ATP-ase (Kuijpers and Bonting, 1970).

In conclusion, we believe that the present data, obtained shortly after animal death, provide a reliable picture of the progressive increase of K^+ concentration in the ampullary endolymph during embryonic development, even though the absolute values in the living animal might be slightly higher. This however does not affect the discussion reported below.

Both single- and double-barrel microelectrode recordings showed that at E9–11 the endolymph is largely immature; K^+ concentration inside the ampulla (8.5 ± 3.2 mM; $n = 12$) was in fact only slightly higher than the expected K^+ concentration in the perilymph—although the K^+ concentration in chicken vestibular perilymph is not known, in cochlear perilymph it was reported to be around 2 mM in chicken embryos (E14–20), and up to 8.1 mM in adult chickens, (Runhaar et al., 1991), versus for example, 1.7 mM in frog vestibular perilymph (Ferrary et al., 1992), 4.2 mM in pigeon cochlear perilymph (Sauer et al., 1999), or 3–4 mM in mammalian cochlear perilymph (reviewed in Ferrary and Sterkers, 1998).

In the chicken embryo, by E9 all the major components of the membranous labyrinth (semicircular canals, ampullae, saccule, utricule, cochlear duct, endolymphatic duct, and endolymphatic sac) have formed, and subsequent morphogenesis from E9 to E18 mainly concerns increasing the overall size of the labyrinth (Bissonnette and Fekete, 1996). Moreover, by E9, vestibular hair cells are already distinguishable from supporting cells by a short stereociliary apparatus. The earliest afferent synapse-like contacts are observed around E7 (Ginzberg and Gilula, 1980). However, the junctional complexes between hair cells and supporting cells are completely developed only by E11 (Meza and Hinojosa, 1987). This is consistent with our finding that endolymphatic K^+ only starts to increase from E12.

The reason for the high-K^+ concentration in the endolymph appears to be that of sustaining the transducer current. At E9–10 chicken embryo hair cells display stereocilia, on which tip links are present (Tilney et al., 1986). Since at E9–10 the largely predominant cation is not K^+, but presumably Na^+, the latter would carry most of the transducer current. Indeed, mechanosensory channels in chicken vestibular hair cells have been shown to display a higher permeability to Na^+ than K^+ (Omori, 1984). Moreover, in the chicken cochlea the mechano-sensory channels already appear functional at E12 (Si et al., 2003). In principle, this Na^+ signal could have a role during early hair cell differentiation; on the other hand, the genesis and maturation of new hair cells in the adult avian occurs in a presumably normal endolymph (Masetto and Correia, 1997).

As the embryo grows the endolymphatic K^+ concentration increases, while total osmolarity is probably maintained by a compensatory decrease in Na^+ concentration. By E16–17 K^+ concentration in the endolymph is above 100 mM; at this time hair cells express a mature-like array of voltage-dependent outward rectifier K channels (Masetto et al., 2000), through which K^+ can leave the cell at the basal pole. The apical-basolateral K^+ current represents the transducer current which is modulated by the natural stimuli, as originally postulated by Davis (1965, 1968). In this way, the depolarization that generates the receptor potential in mature hair cells is produced by K^+, instead of Na^+ or Ca^{2+} as usually occurs in excitable cells. Hair cells are thus relieved from the otherwise necessary trouble of actively pumping out the ions carrying the transducer current, a particularly onerous work for hair cells when considering that even at rest (i.e. in the absence of stimuli) there is a transducer current flowing, which is coupled to spontaneous release of neurotransmitter. In this way the energy expenditure to maintain the gradient for the transducer current is transferred to the dark cells (Wangemann, 1995), which maintain the transepithelial gradient for K^+.

By E19 the K^+ concentration in the endolymph reaches a plateau level, which is presumably maintained after hatching. By the time of hatching, in fact, the chicken inner ear sensory epithelia appear essentially mature (Forge et al., 1997; Masetto et al., 2003).

The process of endolymph development could be at least partially re-created in vitro with the organotypic culture here described. The autonomous ability of the ampullae to secrete the endolymph is not surprising in itself, as it has been shown for the semicircular canal ampullae isolated from the frog (Ferrary et al., 1992, 1995). Here however, quite amazingly, a lumen is re-separated from the extracellular medium by tissue proliferation of the ampullary walls, that seals the lesions produced by the dissection. Meanwhile intra-luminal K^+ concentration begins to rise. This might indicate that by E11 vestibular dark cells have been prompted to produce the endolymph, and are now committed to increase K^+ concentration in the lumen; the ion transporters needed for this task appear to be in place and working.

Histological and therapeutic observations of inner ear pathologies, like for example Ménière's disease or Bartter syndrome (Hebert, 2003), suggest that impaired homeostasis of inner ear fluids is involved. The restoration of a normal condition in these illnesses is not currently possible. However, interfering with endolymph production can improve patients' conditions; for example, the administration of compounds like gentamicin, that disrupt the endolymph-secreting vestibular dark cells, can relieve patients with Ménière's disease from vertiginous attacks (Trine et al., 1995). More recently, it has been shown that in primary cultures of mouse semicircular canal duct epithelial cells, Cl^- channels (Milhaud et al., 2002) and epithelial Na channels (ENaC) are involved in endolymph ion-homeostasis. Modulation of ENaC channels particularly could account for the therapeutic effects of glucocorticoids in the treatment of patients with Ménière's disease (Pondugula et al., 2004). ENaC expression in rat inner ear organs is developmentally regulated (Grunder et al., 2001).

Experimentally, the creation of an in vitro, double compartment system capable of producing endolymph might be effective in studying the effects of different pharmacological agents in the process of endolymph development and homeostasis.

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