Effect of locally applied drugs on the pH of luminal fluid in the endolymphatic sac of guinea pig

VINCENT COULOIGNER,1 MARIE TEIXEIRA,1 PHILIPPE HULIN,2 OLIVIER STERKERS,1 MAURICE BICHARA,1 BRIGITTE ESCOUBET,1 GABRIELLE PLANELLES,2 AND EVELYNE FERRARY1
1Institut National de la Santé et de la Recherche Médicale U.426, Faculté Xavier Bichat, Université Paris 7, 75870 Paris Cedex 18; and 2Institut National de la Santé et de la Recherche Médicale U.467, Faculté Necker Enfants-Malades, Université Paris 5, 75730 Paris Cedex 15, France

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The endolymphatic sac is involved in the regulation of the volume of endolymph by two different mechanisms. The first is endolymph resorption, which has been demonstrated by the endolymphatic hydrops, i.e., the increase in cochleovestibular endolymph volume observed after the experimental destruction of the endolymphatic sac (14). Theoretically, endolymph resorption by the endolymphatic sac should take place in the basal state to compensate the constant secretion of water into the endolymph induced by the hyperosmolality of cochleovestibular endolymph (23). However, indirect measurement of the longitudinal flow of endolymph, i.e., the water flow from cochleovestibular endolymph to the endolymphatic sac lumen, showed that this flow did not differ from zero in the basal state, and it increased when cochlear endolymph was transiently increased (20). The second way by which the endolymphatic sac regulates endolymph volume is by the luminal secretion of osmotically active glycoconjugates. This secretion has been demonstrated in the basal state in most animal species and was stimulated in experimental situations that tend to reduce the volume of endolymph, such as after glycerol administration. In these experimental situations, luminally secreted osmotically active glycoconjugates could retain water in endolymph and thus avoid the collapse of cochleovestibular endolymphatic compartment (27).

In human pathology, the most common cause of dysregulation of endolymph volume is endolymphatic hydrops. This hydrops is known to constitute the anatomic substratum of Ménière’s disease, a syndrome comprising vertigo, hearing loss, and tinnitus, and histologically characterized by an endolymphatic hydrops (11). Malfunction of the endolymphatic sac has been suggested as part of this pathology as lesions of this structure (agenesis, fibrosis, inflammation) have been suggested as part of this pathology as lesions of this structure (agenesis, fibrosis, inflammation) have been observed in some cases (for review, see Ref. 16). Determination of the ionic transport systems involved in both resorptive and secretory functions of the sac may help us to better understand the etiopathogenic mechanisms.
mechanism of Ménière’s disease. It could also facilitate improvements in the medical treatment of this pathology, in particular diuretic treatment that has been widely used on an empirical basis with controversial results (for review, see Ref. 5). Among the diuretics, two drugs, acetazolamide and amiloride, interacting with acid-base transport systems have been reported to improve the evolution of both Ménière’s disease and experimental endolymphatic hydrops (5, 21, 26). For this reason, we investigated endolymphatic sac acid-base transport systems of the guinea pig endolymphatic sac by in vivo measurements of the evolution of the transepithelial potential (ESP) and luminal pH (pH_lum) of the endolymphatic sac after local administration of inhibitors of acid-base transport systems. The results are consistent with active proton secretion in the lumen by an apical vacuolar type H^+ -ATPase and with the involvement of carbonic anhydrase in acid-base net transport.

MATERIALS AND METHODS

Animal Preparation and Endolymphatic Sac Approach

Adult male pigmented guinea pigs (300–400 g body wt, Elevage d’Ardenay, France) were fed with a laboratory chow of constant composition (C15–50, Extralabo Pietrement, Provins, France), and free access to tap water was allowed until the beginning of the experiment. The care and use of the animals had been approved by Ministère de l’Agriculture et de la Forêt (approval number 5521).

Anesthesia was obtained with two successive (in 30 min) intramuscular injections of 2 ml/kg body wt of a mixture of Ketamine (50 mg/ml, Panpharma, Fougeres, France) and Xylazine (2%, Rompun, Bayer, Leverkusen, Germany) (2:1 vol/vol) and was maintained throughout the experiment by injecting half a dose hourly. A heating pad maintained the body temperature between 36.5 and 37.5°C. The animals breathed spontaneously. The animal’s head was fixed in ventral position with a head holder.

The endolymphatic sac was approached through the posterior cerebral fossa under stereomicroscopic observation (Carl Zeiss, Oberkochen, Germany). After suboccipital craniotomy, the dura mater of the posterior fossa was opened, and the endolymphatic sac was approached intradurally following retraction of the hemiserebellum.

pH_lum Measurement

pH_lum was measured with a double-barrelled glass microelectrode (one barrel for the ESP recording; the other for proton electrochemical recording) with an outside tip diameter of ~1 μm. The technique for the construction of these microelectrodes has been described in detail elsewhere (18, 29). One channel was exposed to silane vapours (dimethyltrimethyl-silylamine, Fluka, Sigma) before introducing a droplet of H^+ ion exchanger. Preliminary tests on drug interference with pH sensitivity led us to use the 95297 exchanger, except for ethylisopropylamiloride (EIPA) experimental series for which 95293 exchanger was used (Fluka, Sigma). On the day of the experiment, the conventional barrel of the microelectrode was backfilled with 150 mM NaCl, and the selective channel was backfilled with a solution containing (in mM) 500 KCl, 64.7 KH2PO4, and 85.3 Na2HPO4. The microelectrode tip was immersed for at least 1 h in a Trizma base solution buffered to pH 7.0 with HCl.

The microelectrode was connected via Ag/AgCl electrodes to the input of a high-impedance electrometer (WPI FD 223, World Precision Instruments, Sarasota, FL) whose output was displayed with a pen chart recorder (SRM, Sefram, Paris, France). The electrical circuit was closed by a macroelectrode containing 150 mM NaCl in agarose. pH selectivity was tested in Trizma base/HCl solutions adjusted to various pH values in the range of 6.4–7.4. The slope, S, of the electrodes ranged from 50 to 61 mV per pH unit change. For the experiment, the reference macroelectrode was inserted into the neck muscles, and the tip of the microelectrode was submerged in a superfusing fluid ([in mM] 140 NaCl, 5 KCl, 1 CaCl2, 1 MgCl2, 5 TES, pH 7.4. osmolarity = 280 mosM) delivered at a rate of 5 ml/min over the endolymphatic sac. Zero potential and reference pH (pH_ref) were determined by submerging the microelectrode in the superfusing fluid. The pH_lum was calculated according to the equation pH_lum = pH_ref − (V_E - ESP)/S, where the proton electrochemical recording (V_E) and the ESP were measured by advancing, by means of a micromanipulator (MM3, Narishige, Tokyo, Japan) and under stereomicroscopic observation, the microelectrode into the lumen of the sac and were recorded directly on the chart recorder. At the end of the experiment, the microelectrode was replaced in the reference solution. Results obtained with drifting microelectrodes were rejected.

Local Injections of Various Drugs

The time course of pH_lum under different conditions was studied in 84 animals. The initial pH_lum was measured before injecting 5 μl of either the control or an experimental solution. The solution was slowly injected (taking 5 to 10 min) through a glass micropipette (tip diameter ~5–6 μm) introduced, after penetration of the dura mater, into the area of the extraosseous portion of the endolymphatic sac. The pH_lum was then measured continuously up to 90 min after the end of the injection.

The effect of the control solution ([in mM] 140 NaCl, 5 KCl, 1 CaCl2, 1 MgCl2, 5 TES, pH 7.4) on pH_lum was tested in 17 animals. Nine other separate experimental series were performed in which the injected solutions contained the following: acetazolamide (10^-3 M, n = 10; Diamox, Theraplix, Paris, France), ouabain (10^-3 M, n = 9; Sigma, St. Louis, MO), baflomycin A1 (10^-5 M, n = 8; gift of K. Altendorf, Osnabrück, Germany), Schering 28080 (Sch 28080) (10^-6 M, n = 4; gift from Schering-Plough Research Institute, Kenilworth, NJ), amiloride (10^-4 M, n = 12; Sigma), EIPA (10^-5 M, n = 4; Interchim, Montluçon, France), DIDS (10^-3 M, n = 6; Sigma), triflocin (10^-3 M, n = 9; Lederle), and SITS (10^-3 M, n = 3; Sigma). The drugs were dissolved directly in control solution, except for baflomycin, which was dissolved in DMSO (final dilution 1/1,000 vol/vol). The pH of the injected solution was checked and eventually adjusted to pH 7.4.

Statistical Analysis

Data are expressed as mean ± SE. Except when otherwise stated in the text, analysis of the results was performed by paired t-test for comparison with the initial pH_lum value. Differences were considered significant at P < 0.05. The number of experiments corresponds to the number of injected endolymphatic sacs.
statistical correlation was found between the initial animals (Table 2, one-way analysis of variance). No
lumen positive, ranging from 1.0 to 18.8 mV. No dif-

pression of an acetazolamide-induced disequilibrium
Fig. 2). This observation is best explained by the ap-
pearance of an acetazolamide-induced disequilibrium

When control saline solution was injected, the en-
dolymphatic sac ESP decreased rapidly and had not
determined in blood and endolymphatic sac luminal
fluid from the same animal, no correlation was found
Table 2. Transepithelial potential values (mV) before (initial) and 5, 30, 60, and 90 min after the basolateral injection of drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>n</th>
<th>Initial</th>
<th>5 min</th>
<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17</td>
<td>7.04 ± 0.06</td>
<td>6.99 ± 0.08</td>
<td>7.02 ± 0.07</td>
<td>6.98 ± 0.07</td>
<td>6.95 ± 0.07</td>
</tr>
<tr>
<td>Acetazolamide 10⁻³ M</td>
<td>10</td>
<td>6.92 ± 0.13</td>
<td>6.49 ± 0.13</td>
<td>6.52 ± 0.16</td>
<td>6.56 ± 0.015</td>
<td>6.50 ± 0.14</td>
</tr>
<tr>
<td>Ouabain 10⁻³ M</td>
<td>9</td>
<td>6.86 ± 0.11</td>
<td>6.73 ± 0.10</td>
<td>6.86 ± 0.10</td>
<td>6.94 ± 0.12</td>
<td>7.09 ± 0.10</td>
</tr>
<tr>
<td>Sch 28080 10⁻³ M</td>
<td>4</td>
<td>7.16 ± 0.09</td>
<td>7.10 ± 0.11</td>
<td>7.13 ± 0.07</td>
<td>7.17 ± 0.12</td>
<td>7.13 ± 0.07</td>
</tr>
</tbody>
</table>
| Bafilomycin 10⁻³ M | 8  | 7.06 ± 0.11| 7.11 ± 0.22| 7.45 ± 0.22| 7.46 ± 0.15| 7.33 ± 0.13*
| Amiloride 10⁻¹ M   | 12 | 7.17 ± 0.11| 7.04 ± 0.08| 7.24 ± 0.07| 7.34 ± 0.08| 7.33 ± 0.08|
| EIPA 10⁻⁵ M       | 4  | 7.09 ± 0.18| 6.99 ± 0.08| 7.15 ± 0.09| 7.07 ± 0.16| 7.14 ± 0.12|
| DIDS 10⁻³ M       | 6  | 7.19 ± 0.09| 6.87 ± 0.08| 6.93 ± 0.09| 6.99 ± 0.11| 6.98 ± 0.11*
| Triflocin 10⁻³ M   | 9  | 7.13 ± 0.10| 7.02 ± 0.07| 7.07 ± 0.09| 7.04 ± 0.11| 7.00 ± 0.12|
| SITS 10⁻³ M       | 3  | 7.20 ± 0.09| 7.02 ± 0.06| 7.19 ± 0.03| 7.22 ± 0.07| ND        |

Values are means ± SE. n is the number of experiments. ND, not determined. In this series of experiments, the pH was followed only up to 60 min after the drug injection. Comparison to the control value measured at the same experimental time (Student’s t-test): *P < 0.05.
Effect of ATPases inhibitors on pH lum. Neither ouabain, an inhibitor of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase and of H\textsuperscript{+}-K\textsuperscript{+}-ATPase, nor Sch 28080, a H\textsuperscript{+}-K\textsuperscript{+}-ATPase inhibitor, had any effect (Table 1). The lack of effect of these drugs suggests the absence of involvement of H\textsuperscript{+}-K\textsuperscript{+}-ATPase in luminal acid secretion (9).

By contrast, bafilomycin A\textsubscript{1}, an inhibitor of the vacuolar type H\textsuperscript{+}-ATPase, had induced an increase in pH\textsubscript{lum} 30 min after its injection. Although this increase tended to subside, it was still significant 90 min after the injection (Fig. 3, Table 1).

Effect of Na/H antiporter inhibitors. Both EIPA, a Na/H exchange-specific inhibitor, and amiloride, used at a concentration known to inhibit Na/H exchange, failed to modify the pH\textsubscript{lum} (Table 1).

Effect of anionic transport systems inhibitors. Triflucin and SITS failed to alter the pH\textsubscript{lum}, whereas DIDS induced a rapid (significant as early as 5 min after the injection) and sustained pH decrease (Fig. 4).

DISCUSSION

The purpose of the present in vivo study was to assess the acid-base homeostasis systems of the endolymphatic sac by testing the effects of locally applied drugs on the pH\textsubscript{lum}. Our results suggest a net apical proton secretion involving an apical, vacuolar type H\textsuperscript{+}-ATPase and an intracellular and/or apical membrane-bound carbonic anhydrase.

Endolymphatic Sac Local Drug Delivery

As already reported, experimental injection of solutions into the connective tissue surrounding the endolymphatic sac appears to be a likely route for inner ear gene transfer (33). Regarding the inhibition of ionic transports, local injection allowed us to avoid the major drawbacks of systemic administration, especially the adverse side effects of the injected drugs and shifts in blood pH that could indirectly affect the pH\textsubscript{lum} of the endolymphatic sac. Considering the basolateral site of the injection, the long duration of the experiment, together with the absence of washing of the basolateral interstitial fluid, may have allowed luminal diffusion of the drug and thus precluded any precise localization of the inhibited transport system. Actually, some of the injected drugs, such as bafilomycin, probably acted on apical membrane transporters.

Net Acid Secretion in the Lumen of the Endolymphatic Sac

Positive ESP (+6.1 mV) as well as acidic pH in the lumen (7.06) would drive a paracellular H\textsuperscript{+} absorption, which implies an equivalent transcellular H\textsuperscript{+} secretion in the steady state. Thermodynamic evidence of active proton extrusion from cell to lumen would require direct measurement of cell membrane potential and intracellular pH. However, the effect of bafilomycin on pH\textsubscript{lum} is an indirect but strong argument in favour of H\textsuperscript{+} active transport through the apical cell membrane,
namely an H⁺-ATPase. As bafilomycin caused an increase in pH, reaching 7.46, a pH that would correspond to a passive H⁺ transepithelial distribution, a vacuolar H⁺-ATPase was probably responsible for at least most of the transepithelial H⁺ gradient in the endolymphatic sac. In view of the high concentrations of bafilomycin used in the present study, the specificity of this drug on vacuolar H⁺-ATPase could be questioned. Nevertheless, in vitro studies showed that at 10⁻⁵ M, bafilomycin A₁ is still highly specific of vacuolar H⁺-ATPase (4). Moreover, once injected, bafilomycin was diluted in the basolateral compartment of the sac, so that its concentration at the site of its target transporter was probably <10⁻⁵ M. Finally, our functional observations in favour of an apical vacuolar H⁺-ATPase are consistent with immunohistochemical results (22).

The absence of any effect of amiloride and EIPA on the pHeₙₚ of the endolymphatic sac is an additional argument for the predominant role of the H⁺-ATPase in the maintenance of the pHeₙₚ. The presence of an apical Na⁺/H⁺ antiporter has been postulated, in view of the decrease in the ESP after amiloride application (8) and the inhibition of acid-load recovery by amiloride on epithelial cells isolated from the endolymphatic sac (32). Nevertheless, under the present experimental conditions, the maintenance of a stable pHeₙₚ after amiloride and EIPA injections suggests that the Na/H transport system may not be involved in the generation of the transepithelial acid gradient.

Acetazolamide, a drug known to inhibit carbonic anhydrase, caused a rapid and sustained decrease in the pHeₙₚ. This luminal acidification might have been the result of various effects of acetazolamide, including impairment of the acid-base transport systems. However, the most straightforward explanation is that acetazolamide induced an acid disequilibrium pHeₙₚ due to the inhibition of carbonic anhydrase in functional contact with the luminal fluid. A similar phenomenon takes place in the proximal tubule in the kidney, where acetazolamide or benzolamide induced an acid disequilibrium pHeₙₚ (19, 30). Consistent with this hypothesis, an ultrastructural study showed that carbonic anhydrase was not only located in the cytoplasm, but it was also bound to apical and basolateral membranes of the mitochondria-rich cells of the endolymphatic sac (25).

**Net Reabsorption of HCO₃⁻ or Equivalent Species from Lumen to Endolymphatic Sac**

Net proton secretion into the lumen of the sac is expected to be coupled to a transepithelial reabsorption of HCO₃⁻ or equivalent species. Yet the present study failed to detect any effect of various inhibitors of HCO₃⁻ transport systems. Inhibitors of Cl⁻/HCO₃⁻ exchanger and more generally of anion transport systems, including Na⁺/HCO₃⁻ cotransport, such as SITS, triflucin, and DIDS (2, 7), did not cause alkalinization of the luminal fluid. On the contrary, DIDS induced a decrease in the pHeₙₚ. At the concentration used in the present study, DIDS has been reported to inhibit Cl⁻ channels (15) and paradoxically to activate ion transport systems such as nonselective cationic channels (10) or Iₖ, K channels (6). Thus although DIDS-induced luminal acidification may result from the inhibition of an apical base-equivalent transport mechanism on the apical membrane, such as a Cl⁻/HCO₃⁻ exchanger, no firm conclusion can be drawn from the present observations. Further studies with molecular tools are necessary to elucidate the site of action of DIDS.

**Correlation Between ESP**

In the present study, no correlation was found in basal conditions (before drug administration) between endolymphatic sac ESP and pHeₙₚ. Moreover, in the control group, evolution of ESP was biphasic, with an initial decrease followed by partial recovery, whereas pHeₙₚ remained stable throughout the experiment. Thus although the decrease in the ESP by bafilomycin suggested the participation of this ATPase in the genesis of the ESP, other transport systems, such as Na⁺ and K conductances and/or Na⁺-K⁺-2Cl⁻ cotransport, probably play a major role in this process (17, 28, 31).

**Role of Luminal Net Acid Secretion in Endolymph Homeostasis and Clinical Involvements**

The acidity of the luminal fluid in the endolymphatic sac relative to blood physiological pH probably contributes to endolymph homeostasis in different ways. First, it might modify the activity of aquaporins involved in endolymph resorption. Indeed, the osmotic water permeability of AQP3, an aquaporin whose mRNA has been detected by RT-PCR in rat endolymphatic sac (1), is inhibited by low extracellular pH (34). Second, low pHeₙₚ might activate the degradation of luminal precipitates of osmotically active luminal glycoconjugates by free-floating macrophages (3, 12).

Mutations of the B₁ subunit of the vacuolar H⁺-ATPase have recently been shown to lead to sensorineural hearing impairment associated with distal tubular acidosis (13). The B₁ subunit has been localized in the endolymphatic sac by immunohistochemistry (13). The specific involvement of vacuolar H⁺-ATPase of the endolymphatic sac in this genetic deafness remains to be determined.

**Perspectives**

Our results are consistent with active luminal proton secretion by means of an apical bafilomycin A₁-sensitive vacuolar H⁺-ATPase and with the presence of intracellular and membrane-bound carbonic anhydrases. The resulting luminal acidity relative to blood pH probably participates in the homeostasis of endolymph by interacting with aquaporins and/or with the degradation of luminal glycoconjugates by macrophages. This regulation is a tempting explanation for the contrary effects of acetazolamide in endolymph homeostasis, depending on whether the endolymphatic sac is
functional (21, 26). Indeed, after destruction of the sac, this diuretic prevented the development of endolymphatic hydrops (21), whereas it induced mild endolymphatic hydrops when the endolymphatic sac was intact (26). On a more general standpoint, one must take into account the acid-base transport systems of the endolymphatic sac when predicting or interpreting the efficacy on Ménière’s disease of diuretics that interact with the acid-base balance. The role of the endolymphatic sac apical $H^+$-ATPase in the pathophysiology of genetic deafness resulting from mutations of the B1 subunit of this pump remains to be determined.

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