Open Access

Regulation of Cochlear Fluids pH: (II) The Role of Bicarbonate

Masaaki Suzuki[*], Tadashi Wakayama and Ryosuke Kotani

Department of Otolaryngology, Teikyo University Chiba Medical Center, Chiba, Japan

Abstract: Bicarbonate provides the dominant pH buffer of the cochlear fluids. In the present study, perilymph bicarbonate concentration was manipulated during which endocochlear potential, cochlear fluids pH and cochlear sensitivity to sounds were monitored. Perilymphatic perfusion of medium containing elevated HCO_3^-, at higher pH than normal, produced a decrease in endolymph pH. Similarly, perfusion of nominally HCO_3^--free solution, with lower pH than normal, produced an increase in endolymph pH. When pH changes were induced with HEPES-buffered media, endolymphatic and perilymphatic pH changed in the same direction. The observed endolymph pH changes induced by perilymphatic HCO_3^- manipulations therefore depend on HCO_3^-, rather than pH changes. These findings can be explained by the generation of localized changes of CO_2 in the cochlear fluids associated with bicarbonate homeostasis. These data demonstrate some key properties of the CO_2/HCO_3^- buffer system and suggest the likely role for carbonic anhydrase in tissues of the lateral wall. In practical terms, the data also suggest that the pH stability of inner ear fluids cannot be adequately maintained when bicarbonate-altered media are perfused, especially when pH buffers other than HCO_3^- are used.

Keywords: Cochlear fluids pH, endocochlear potential, perilymph bicarbonate.

INTRODUCTION

Although it is known that cochlear fluids pH is dominated by the CO_2/HCO_3^- buffer system, there are only limited data on the characteristics of pH regulation in the cochlea and no systematic studies of how cochlear fluids pH depends on HCO_3^- level. In a previous study, it was shown that the pH of cochlear fluids depended on respiratory CO_2 status. Elevation of arterial CO_2 (as indicated by end-tidal CO_2 measurement) produced acidification of endolymph and perilymph, in accordance with the known CO_2-dependence of the HCO_3^- buffer system (reviewed by Hruska [1]). In the present study, we have investigated the influence of changes in HCO_3^- concentration, while carefully maintaining a stable respiratory CO_2 status. In any solution, changes of HCO_3^- concentration result in pH changes, with pH increasing as HCO_3^- is increased [2]. The HCO_3^- concentration in cochlear fluids is difficult to measure directly, since it was found that HCO_3^- ion exchangers available for ion-selective electrodes are far more selective for carbonate than HCO_3^- [3]. For cochlear perfusion, many groups use a medium similar to that described by Konishi and Kelsey [4], containing 12 mM HCO_3^-, which is well below the presumed normal physiologic level of 20 to 25 mM in guinea pigs [5, 6]. A few investigators have used perfusion media incorporating non-HCO_3^- buffers and low HCO_3^- content. Since HCO_3^- represents approximately 20% of the anion content of endolymph, homeostasis of HCO_3^- likely plays a significant role in maintaining normal endolymph status. HCO_3^- ion movements are likely to contribute directly to composition, osmolarity and volume changes of endolymph during experimental treatments or pathologic situations. In addition, pH changed associated with HCO_3^- disturbances are likely to modulate the transport of other ions, such as the transport of K^+ into endolymph [7].

METHODS

This study used 19 pigmented guinea pigs weighing 300-500 g, anesthetized with a combination of sodium pentobarbital (25 mg/kg i.p.) and Innovar-Vet (O.35 mllkg Lm.). (lnnovarVet contains 0.4 mg/ml fentanyl and 20 mg/ml droperidol). Sodium pentobarbital was supplemented as required to maintain deep surgical anesthesia *via* an intravenous line in the left external jugular vein. Body temperature was maintained at 38-39 °C with a thermistor-controlled heating pad. The trachea was cannulated and, prior to recordings, animals were immobilized with gallamine triethiodide (2-3 mg/kg) and artificially ventilated. Expired CO_2 was monitored using a Puritan-Bennett Datex CO_2 analyzer, designed for use with small ventilation volumes and equipped with peak-hold circuitry. Throughout the recording period, end-tidal respiratory CO_2 was maintained close to 5% (38 mm Hg) by periodically adjusting the tidal volume of the respirator as necessary. The head of the animal was secured in a rigid head-holder and the right cochlea was exposed by a ventrolateral approach.

The method and the recording system for the microelectrodes have been described elsewhere [8]. In brief, all measurements of endolymph were made in the second cochlear tum through small access fenestras made in the lateral wall. The fenestras, which were 30 to 50 micrometers in diameter were made with a fine pick after first thinning the bone with a flap knife. In the case of endolymphatic

*Address correspondence to this author at the Department of Otolaryngology, Teikyo University Chiba Medical Center, 3426-3, Anesaki, Ichihara, Chiba, 299-0111, Japan; Tel: +81-436-62-1211; Fax: +81-436-61-8474; E-mail: suzukima@med.teikyo-u.ac.jp

measurements, endocochlear potential (EP) was recorded from the reference barrel of the ion electrode. The pH of endolymph and scala vestibuli perilymph were measured by double-barreled pH-selective electrodes. Double-barreled electrodes were made from borosilicate capillary and one barrel was silanized by exposure to dimethyldichlorosilane (Sigma, St. Louis, USA) vapor and the tip was broken to 2-3 micrometers in diameter. The ion barrel of electrodes was filled with 40 mM KH_2PO_4, 23 mM NaOH and 15 mM NaCl [9] and the potential barrel was filled with 500 mM KCl. The pH-sensitive exchanger for this electrode was Fluka 95297 (Fluka Chemical, Ronkonkoma, NY, USA), a small column of which was drawn into the ion barrel by suction. All electrodes were calibrated before and after use in commercially available buffer solutions at pH 8.00, pH 7.00 and pH 6.00 (Fisher Scientific, Pittsburgh, PA, USA) at 38°C. The mean response slope of the H^+ electrodes used in this study was 50.5 mV/decade (SD 1.66, n=29). Potentials were recorded from ion electrodes under computer control using digital meters equipped with IEEE-488 interfaces.

The perilymphatic space was perfused with solution at a rate of 5 microliter/min. Perfusate entered the basal tum of scala tympani through a double-barreled pipette. A bead of paint on the perfusion pipette provided a gasket to minimize leakage at the injection site One Perfusion Pump (Harvard model PHD 2000) and one perfusion pipette barrel was used for each solution perfused. The outlet for perfusate was made in the basal tum of scala vestibuli. Accumulating fluid was continually removed from the bulla by a suction tubing placed behind the cochlear apex. The composition of solutions perfused are given in Table **1**. Solutions were pH buffered with either HCO_3^- or HEPES. For each individual experiment, all perfusions utilized the same buffer and a maximum of two treatments were performed. The osmolarity of these solutions was 292 -295 mOsm. All solutions were pre-equilibrated with CO_2 by bubbling with 5% CO_2 in air for 20 min prior to perfusion. After bubbling, the pH of the HCO_3^- buffered control medium was averaged 7.29 (SD 0.08, n= 9). HEPES-buffered control medium was adjusted to pH 7.4.

Whole nerve action potential (AP) thresholds were monitored from an Ag/AgCl ball electrode placed on the otic capsule close to the round window membrane. AP thresholds were tracked throughout the experiment using an automated procedure according to procedures detailed in Salt and Vora [10]. For each threshold measure, stimulus level was increased in 10 dB steps until a suprathreshold response was detected, and then decreased in 5 dB steps until the response amplitude fell below the threshold value. Interpolation was used to establish the sound level at which a response of criterion amplitude would have been generated. For this study, a 20 microvolt criterion was used to establish threshold, which is a higher criterion than we normally use to ensure that responses could be clearly resolved during periods of perfusion.

Data are presented as the mean and the standard deviation (SD) of the number (n) of observations given. All statistical analysis was performed using the Statistical Package for Social Science, version 11.01 (SPSS Inc., Chicago, IL). The study procedures were in accordance with the standards set forth in the eighth edition of Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences, The National Academies Press, Washington, DC, USA.

RESULTS

One issue which this study had to deal with was that cochlear perfusion with control solutions induced systematic changes of cochlear fluid pH and EP in the second turn, and of AP threshold sensitivity. The changes induced by the onset of perfusion with HCO_3^--buffered or HEPES-buffered control solutions are summarized in Fig. (**1**). With HCO_3^--buffered medium, endolymph pH was always increased by the perfusion, the mean increase being 0.10 (SD 0.08, n= 9). The pH measured in SV typically underwent a slight initial decrease, followed by an increase which averaged 0.09 (SD 0.05, n= 5). EP showed a characteristic decrease at the onset of perfusion, followed by a slow increase and AP thresholds were raised by approximately 10 dB.

With the onset of perfusion of HEPES-buffered control medium, endolymph pH changes were significantly larger than those with HCO_3^--buffer (Anova, $p < 0.05$). With HEPES-buffered solution, endolymph pH increased by a mean value of 0.22 (+/-0.12, n= 9). Perilymphatic pH changes were markedly different with HEPES compared with HCO_3^- buffer. With HEPES, perilymph pH showed a rapid acidification to a value well below that of the perfusate

Table 1.   Composition of solutions perfused in this study.

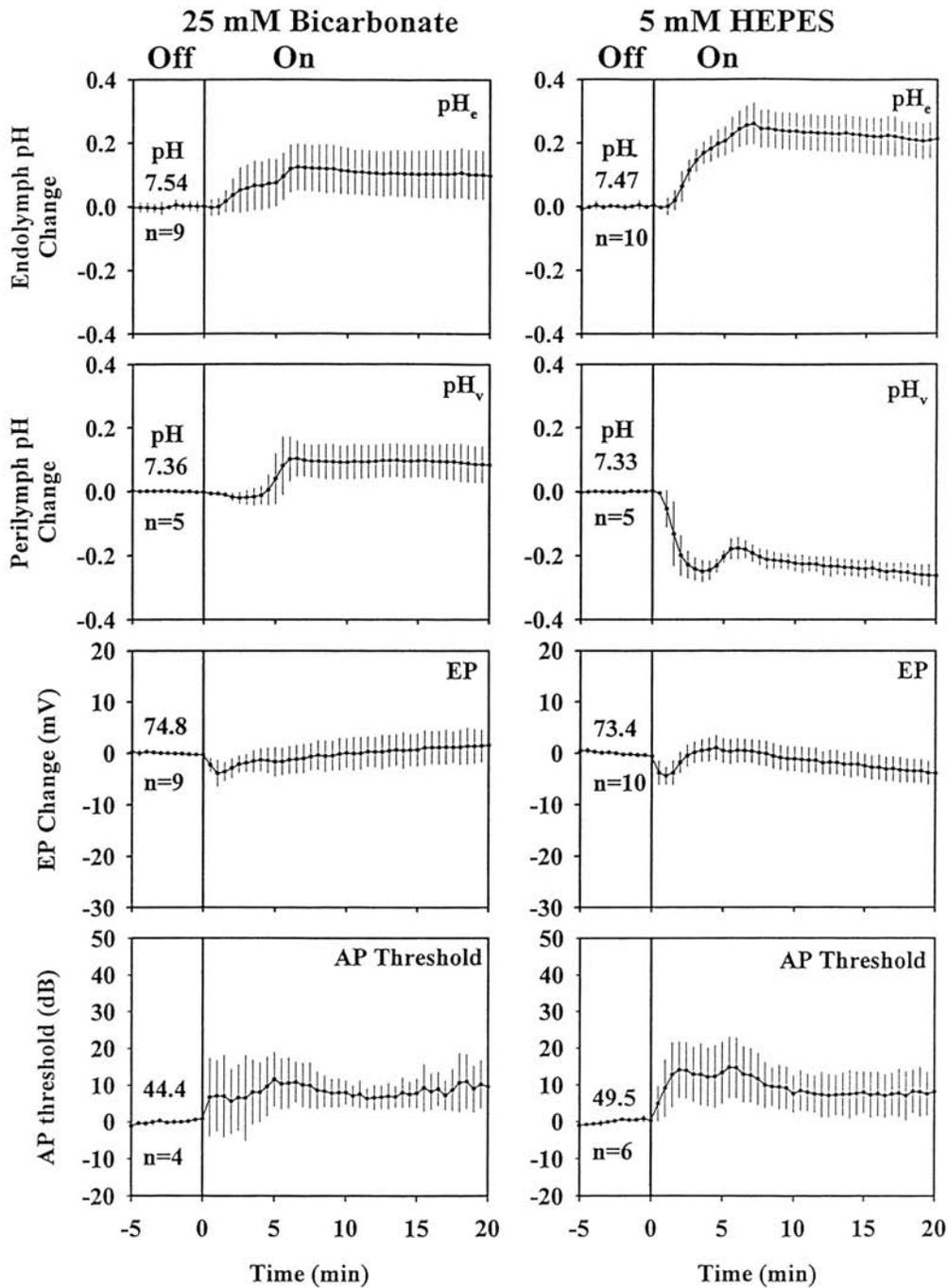| Component (mM)	Normal, Bicarbonate-Buffered	HEPES-Buffered	Hig-HCO_3^- (50 mM)	Low-HCO_3^- (0 mM)
NaCl	122	142	97	147
KCl	3.5	3.5	3.5	3.5
$NaHCO_3$	25	0	50	0
$CaCl_2$	1.3	1.3	1.3	1.3
$MgCl_2$	1	1	1	1
NaH_2PO_4	0.75	0.75	0.75	0.75
D-glucose	11	1	11	11
Na-HEPES	-	5	-	-

Fig. (1). Effects of perfusion onset with bicarbonate (HCO_3^-) or HEPES-buffered (HCO_3^--free) media on endolymph pH, scala vestibuli perilymph pH, endocochlear potential (EP) and cochlear action potential (AP) thresholds in response to 8kHz tone bursts. The heavy vertical line at zero time indicates perfusion onset. The average baseline value and the number of experiments included in the average are indicated. Bars indicate the standard deviation of the mean value.

and the acidification was sustained throughout the perfusion. A notable difference between the effects of HCO_3^- and HEPES solutions, was that for HCO_3^- endolymphatic and

perilymphatic pH changes were not significantly different (Anova, $p > 0.5$) while for HEPES-buffered solution the endolymphatic changes were significantly greater than those

seen in perilymph (Anova, $p< 0.05$). In terms of functional changes, the EP tended to decline with HEPES-buffered solution, compared to the slow increase seen with HCO_3^-. The EP at the end of the perfusion period was significantly lower for the HEPES-buffer (t-test, $p= 0.003$). In contrast, the AP threshold elevations were not significantly different during perfusion of HEPES and HCO_3^- buffered solutions. The pH changes induced by control perfusions are thought to be at least partially induced by mechanical disturbances. We therefore minimized mechanically-induced effects by continually perfusing the cochlea and switching solutions between control and altered media. The effects of varying HCO_3^- concentration by this paradigm are summarized in Fig. (**2**). For a nominally HCO_3^--free solution, which was only buffered to a small extent by PO_4^-, the pH measured after CO_2 equilibration averaged 4.25 (SD 0.4, n= 4). When low- HCO_3^- solution was perfused (Fig. **2**, left panels), perilymph pH was reduced, as expected, but endolymph pH was found to increase. EP showed a transient increase and AP threshold changes were not remarkable. Induced changes were reversible on return to 25 mM HCO_3^- The pH of the elevated HCO_3^- (50 mM) solution averaged 7.58 (SD 0.1, n=

5) after CO_2 equilibration. With elevated-HCO_3^- perfusion, perilymph pH was increased but endolymph pH decreased. Associated with the acidification of endolymph, EP was systematically decreased and AP thresholds were elevated. In an attempt to establish the origins of HCO_3^--induced endolymphatic changes, we induced similar pH changes· while maintaining HCO_3^- level unchanged. The results of pH changes induced by nominally HCO_3^--free, HEPES-buffered solutions, are shown in Fig. (**3**). Solutions were perfused at pH 6.0 and 8.0, values intended to generate comparable perilymphatic pH change. Perfusion of solution with pH 6 resulted in decreases in both perilymph and endolymph pH, a result which is quite different from when pH change was induced by HCO_3^- manipulation. Associated with the acidification of endolymph was a decrease in EP and an elevation of AP thresholds. Perfusion of HEPES-buffered solution at pH 8.0 increased the pH measured in perilymph and endolymph, although the measured changes were smaller than anticipated due to the acidification of perilymph associated with HEPES-buffered perfusion. The pH elevations had only minor effects on EP and AP thresholds.
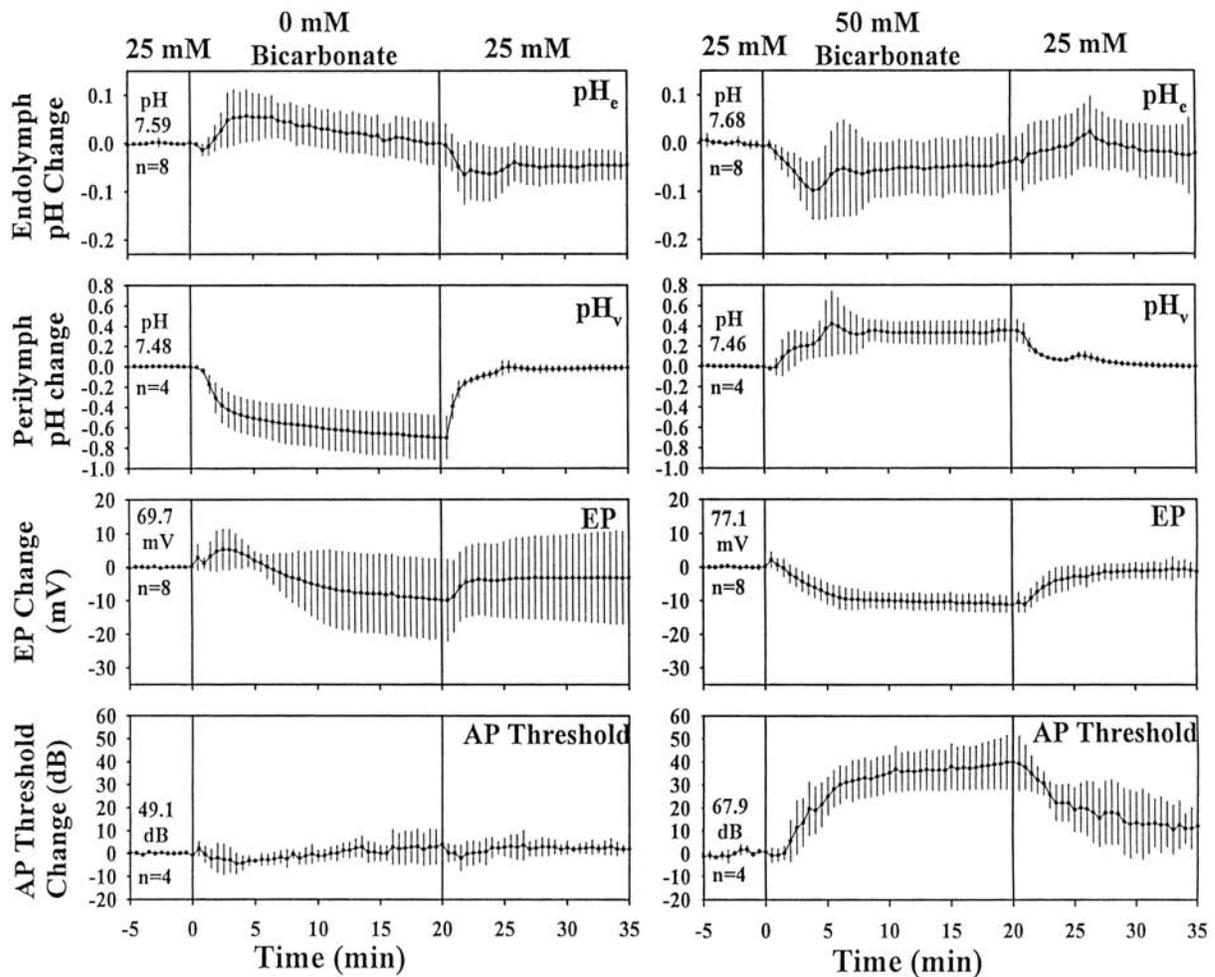

Fig. (2). Effect of perilymphatic bicarbonate (HCO_3^-) changes on the cochlear pH and tone-evoked cochlear responses. Vertical lines indicate the beginning and end of perfusion with solution containing nominally 0 mM HCO_3^- (left side) or 50 mM HCO_3^- (right side). Bars indicate standard deviation. In the periods before and after perfusion with the specified solution, the cochlea was perfused with a control solution containing 25 mM HCO_3^-. Perfusion with altered- HCO_3^- solutions produced oppositely-directed pH changes in endolymph and perilymph.
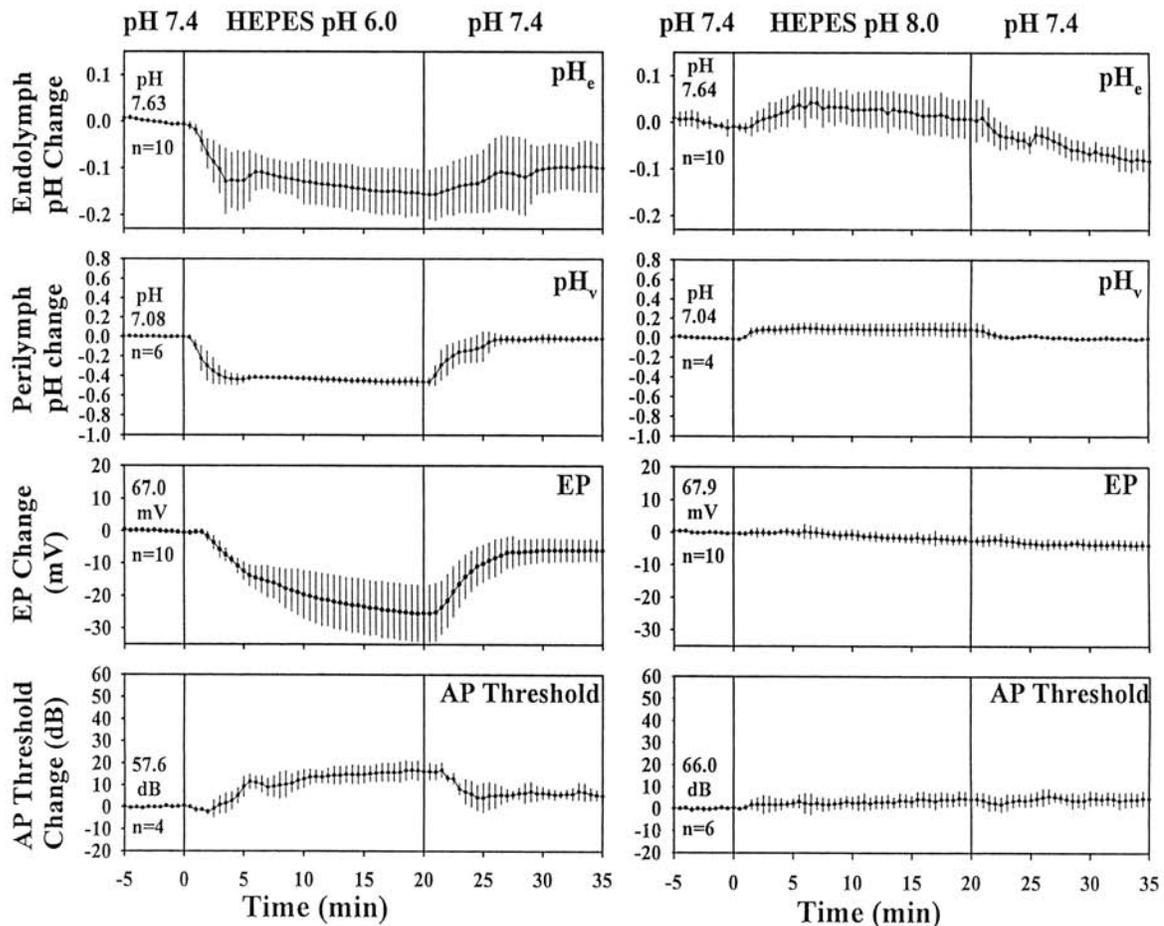
Fig. (3). Effect of perilymphatic pH changes induced by HEPES-buffered (HCO_3^--free) media at pH 6.0 (left) or pH 8.0 (right). Baseline values of each parameter and the number of experiments are given for each curve. Bars indicate standard deviation.

The differences between endolymph and perilymph pH changes induced by HCO_3^- and HEPES-buffered solutions are summarized in Fig. (**4**). Values are the pH change averaged over the time period from 2 to 10 minutes after the start of the altered-composition perfusate. For the HCO_3^- buffer, endolymph and perilymph pH changes were consistently oppositely directed. In contrast, for the HEPES buffered solutions, endolymphatic pH changes were similarly-directed, though smaller than those induced in perilymph.

DISCUSSION

An interesting finding of this study was that endolymph pH changes were induced by HCO_3^- manipulations which were oppositely-directed to those measured in perilymph. This occurred only when HCO_3^- concentration changed, but not when comparable pH changes were induced under constant, HCO_3^--free conditions. The oppositely-directed endolymphatic pH change with perilymphatic HCO_3^- manipulation must therefore result from the HCO_3^-, rather than the pH change. This finding cannot be explained by a mechanism of perilymphatic pH homeostasis mediated by acid or HCO_3^- transport between endolymph and perilymph. In this case, where ions are simply transported, the pH

changes in endolymph and perilymph should be in the same direction. On the other hand, the observations are readily explained if mechanisms likely to underlie HCO_3^- homeostasis are considered. In the case of a reduction in HCO_3^-, the equilibrium between CO_2 and HCO_3^-, catalyzed by carbonic anhydrase, will be skewed in the direction whereby CO_2 will be converted to HCO_3^- and H^+. This would result in a decrease in the steady-state CO_2 level in the perilymph. Since CO_2 readily passes through membranous boundaries, this would in turn decrease the CO_2 level in endolymph, thereby resulting in an alkalinization. Similarly, in the elevated HCO_3^- condition, the restoration of normal HCO_3^- by the addition of acid, would result in a release of CO_2 and subsequent acidification of endolymph. This explanation is consistent with our previous study in which endolymph pH was shown to be highly sensitive to the prevailing CO_2 level [2]. It can be calculated that a relatively small CO_2 change, such as a reduction from 5% to 4% (i.e. a change of 7.6 mmHg), would be sufficient to induce the measured endolymph pH changes, if we can assume that endolymph HCO_3^- remains constant. Endolymph HCO_3^- cannot be readily measured by ion-selective electrode as the commercially-available exchanger which was marketed as HCO_3^--selective has now been shown to be preferentially
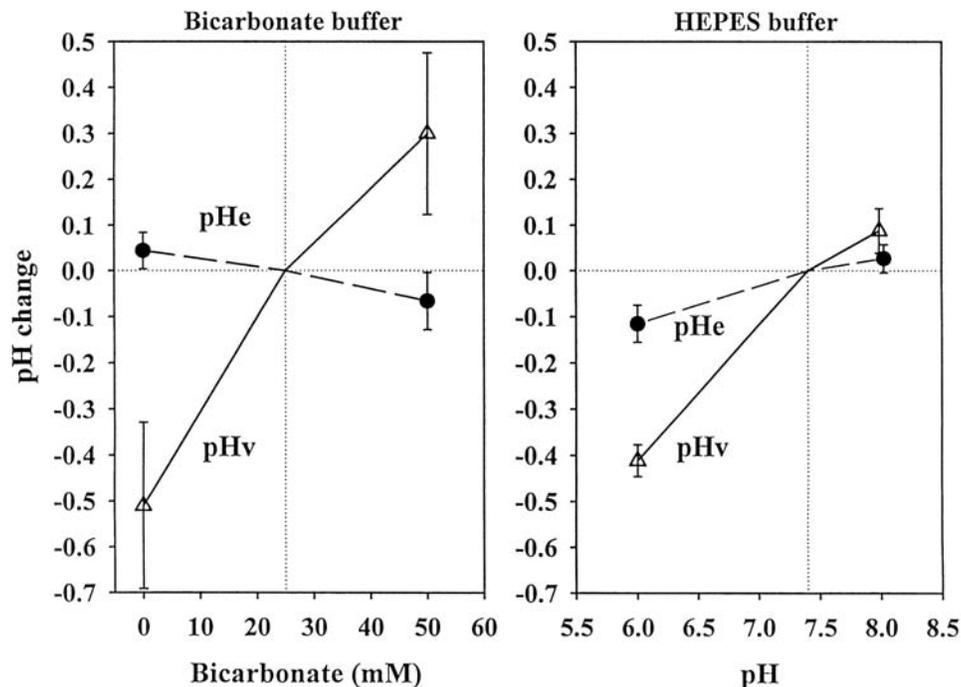
Fig. (4). Summary of endolymphatic (pH_e, dotted lines) and perilymphatic (pH_v, solid lines) pH changes induced when HCO_3^--buffered or HEPES-buffered (HCO_3^--free) media are perfused. Data points are the pH values averaged over the time period from 2 to 10 minutes after perfusion onset. With HCO_3^- buffers, endolymphatic pH changes are oppositely directed to those induced in perilymph. With HEPES-buffered media, endolymphatic anc perilymphatic pH changes are in the same direction. The number of measurements in each condition are shown in Figs. (**2**, **3**).

selective to carbonate (CO_3^{2-}) and may thus give misleading results [3, 11]. The technique of cochlear perfusion is widely regarded as benign, in which control perfusions of the perilymphatic space are believed to have little influence on cochlear function. This impression has partly arisen because many investigators have not measured function during perfusion, but instead make measurements some time after perfusion ends [12-14]. In studies where cochlear responses are measured during perfusion, small decreases in sound-induced responses (cochlear microphonic or action potential amplitudes) and fluctuations of the endocochlear potential are generally observed [4, 15]. The EP changes and AP thresholds we observed at the onset of control (25 mM), HCO_3^--buffered solution are consistent with these previously-published findings. We have further found that significant disturbance of cochlear fluids pH occurs with perfusion. With HCO_3^--buffered media, we systematically observed a substantial alkalinization of endolymph in the second turn at the onset. With HEPES-buffered solution, the alkalinization of endolymph was even greater, even though perilymph became progressively more acidotic with time. The marked acidosis of perilymph is consistent with a high rate of acid generation, presumably derived from CO_2, in which HCO_3^- and H^+ are generated in equimolar quantitites. Similarly, the marked elevation of endolymph pH with HEPES-buffered perfusion onset is a consequence of the mechanisms compensating for the reduced HCO_3^- reducing the available CO_2 as discussed above. One conclusion from these observations is that it is not feasible to maintain pH

stability of the inner ear *in vivo* when HCO_3^--free solutions are employed.

There are a number of possible origins of perfusion-induced pH changes. One likely factor is a perfusion-induced mechanical disturbance of the cochlear partition, influencing currents passing through the boundary tissues and thereby affecting ion movements [16]. Salt & DeMott have demonstrated that perilymphatic perfusion at 5 ul/min induces a sustained basally-directed displacement of endolymph by approximately 0.4 mm, which only returns when perfusion ceases [17]. If the displacement of tissues is sustained throughout the perfusion, as these data suggest, it is likely that sustained changes in ion transport would be induced. The possibility thus exists that-mechanical disturbance may affect ion transport, giving rise to pH changes which *in vitro* studies have demonstrated may in turn give rise to further ion transport changes [7].

An important conclusion from these studies is that the pH of cochlear fluids does not appear to be tightly regulated by the tissues responsible for endolymph and perilymph homeostasis. The high mobility of CO_2 across tissue boundaries would limit the degree of local control of pH in extracellular compartments. In contrast, our results suggest that HCO_3^- homeostasis appears to be of high importance in the intact ear, which would indirectly lead to pH regulation. Since HCO_3^- plays a significant role as a component of endolymph and perilymph, maintenance of fluid HCO_3^- may accomplish both electrolyte and pH homeostasis.

In consideration of the EP changes associated with HCO_3^- and pH manipulations, the two treatments which acidified endolymph (50 mM HCO_3^-, HEPES at pH 6) produced immediate decreases of EP, while treatments which alkalinized endolymph produced no change or small increases in EP. These findings contrast with those induced by CO_2 manipulations in our prior study in which an elevation of CO_2 caused acidosis of cochlear fluids and an increase in EP [2]. In the vascular-perfused preparation, Arakawa *et al*. found EP to be relatively insensitive to vascular HCO_3^- changes, but highly sensitive to vascular CO_2 changes [18]. Low CO_2 tensions induced EP decreases and high CO_2 tensions induced transient EP increases followed by a decline. In the present, perilymphatically-perfused preparation, tissues closely associated with the vascular compartment, such as marginal cells, are less likely to be affected by the experimental manipulations. The study of Wangemann *et al*. found that vestibular dark cells were significantly more sensitive to pH changes applied to the basolateral side compared to the apical side of the epithelium [7]. Thus, it is likely that the difference between experimental findings arises from different tissues being affected by the experimental treatments. This is consistent with there not being a single cell type (such as the marginal cells) involved in EP generation which is CO_2-sensitive, but rather multiple cell types, each with degrees of pH or CO_2 sensitivity which can all affect the circulating current path and hence the EP.

In conclusion, these studies show that CO_2 and HCO_3^- each play an important role in establishing cochlear fluids pH and may thereby influence many aspects of ion transport and cochlear function. As HCO_3^- represents a significant component of both endolymph and perilymph, it is important to detail the mechanisms underlying its homeostasis, in order to understand how the cochlear fluids are maintained in normal and pathological states.

CONFLICT OF INTEREST

This was not an industry-supported study. Authors have no conflicts of interest to declare in relation to the subject matter of this manuscript.

ACKNOWLEDGEMENTS

REFERENCES

[1]   Hruska K. The kidney and body fluids in health and disease. In: Klahr S, Ed. Pathophysiology of acid-base metabolism. New York: Plenum Press 1983; pp. 223-67.

[2]   Suzuki M, Salt AN. Regulation of cochlear fluids pH: (l) The role of carbon dioxide. TOOTORJ 2014 (in review: companion paper)

[3]   Wietasch K, Kraig RP. Carbonic acid buffer species measured in real time with an intracellular microelectrode array. Am J Physiol 1991; 261: R760-65.

[4]   Konishi T, Kelsey E. Effect of potassium deficiency on cochlear potentials and cation contents of the endolymph. Acta Otolayryngol 1973; 76: 410-8.

[5]   Ikeda K, Kusakari J, Takasaka T, Saito Y. Early effects of acetazolamide on ionic activities of the guinea pig endolymph: Evidence for active function of carbonic anhydrase in the cochlea. Hear Res 1987; 31: 211-6.

[6]   Isaacs JH Jr, Pessah N, Merwin GE, Maren TH. Lack of effect of carbonic anhydrase inhibition on direct measurements of endolymph bicarbonate. Ann Otol Rhinol Laryngol 1989; 98: 209-12.

[7]   Wangemann P, Liu J, Shiga N. The pH-sensitivity of transepithelial K^+ transport in vestibular dark cells. J Membrane Biol 1995; 147: 255-62.

[8]   Suzuki M, Kikuchi T, Ikeda K. Endocochlear potential and endolymphatic K changes induced by gap junction blockers. Acta Otolaryngol 2004; 124: 902-6.

[9]   Ammann D, Lanter F, Steiner RA, Schulthess P, Shijo Y, Simon W. Neutral carrier based hydrogen ion selective microelectrode for extra-and intracellular studies. Anal Chern 1981; 53: 2267-9.

[10]   Salt AN, Vora AR. Cochlear threshold assessment using tone-derived action potentials. Audiology 1990; 29: l35-45.

[11]   Chesler M, Chen JC, Kraig RP. Determination of extracellular bicarbonate and carbon' dioxide concentrations in brain slices using carbonate and pH-selective microelectrodes. Neurosci Meth 1994; 53: 129-36.

[12]   Bobbin RP, Fallon M, Kujawa SG. Magnitude of negative summating potential varies with perilymph calcium levels. Hear Res 1991; 56: 101-10.

[13]   Kujawa SG, Fallon M, Bobbin RP. ATP antagonists cibacron blue, basilen blue and suramin alter sound-evoked responses of the cochlea and auditory nerve. Hear Res 1994; 78: 181-8.

[14]   Klis SF, Smoorenberg GF. Osmotically induced pressure difference in the cochlea and its effect on cochlear potentials. Hear Res 1994; 75: 114-20.

[15]   Salt AN, Stopp PE. The effect of raising scala tympani potassium concentration on ton-induced cochlear responses of the guinea pig. Exp Brain Res 1979; 36: 87-98.

[16]   Zidanic M, Brownell WE. Fine structure of the intracochlear potential field. I. The silent current. Biophys J 1990; 57: 1253-68.

[17]   Salt AN, DeMott JE. Longitudinal endolymph movements induced by perilymphatic injections. Hear Res 1998; 123: 137-47.

[18]   Arakawa E, Marcus DC, Thalmann R. Dependence of endocochlear potential on vascular pH. Hear Res 1987; 31: 1-7.