Effects of $\text{CO}_2/\text{HCO}_3^-$ in Perilymph on the Endocochlear Potential in Guinea Pigs

Yoshitsugu NIMURA², Yoshiaki MORI¹, Takaki INUI², Yoshiro SOHMA¹, Hiroshi TAKENAKA²,
and Takahiro KUBOTA¹

Departments of ¹Physiology and ²Otolaryngology, Osaka Medical College, Osaka, 569-8686 Japan

Abstract: The effect of $\text{CO}_2/\text{HCO}_3^-$ on the endocochlear potential (EP) was examined by using both ion-selective and conventional microelectrodes and the endolymphatic or perilymphatic perfusion technique. The main findings were as follows: (i) A decrease in the EP from $\sim +75$ to $\sim +35$ mV was produced by perilymphatic perfusion with $\text{CO}_2/\text{HCO}_3^-$ -free solution, which decrease was accompanied by an increase in the endolymphatic pH (ΔpH_e , ~ 0.4). (ii) Perilymphatic perfusion with a solution containing 20 mM NH_4Cl produced a decrease in the EP (ΔEP , ~ 20 mV) with an increase in the pH_e (ΔpH_e , ~ 0.2), whereas switching the perfusion solution from the NH_4Cl solution to a 5% $\text{CO}_2/25$ mM HCO_3^- solution produced a gradual increase in the EP to the control level with the concomitant recovery of the pH_e . (iii) The

perfusion with a solution of high or low HCO_3^- with a constant CO_2 level within 10 min produced no significant changes in the EP. (iv) Perfusion of the perilymph with 10 $\mu\text{g}/\text{ml}$ nifedipine suppressed the transient asphyxia-induced decrease in EP slightly, but not significantly. (v) By contrast, the administration of 1 $\mu\text{g}/\text{ml}$ nifedipine via the endolymph inhibited significantly the reduction in the EP induced by transient asphyxia or perilymphatic perfusion with $\text{CO}_2/\text{HCO}_3^-$ -free or 20 mM NH_4Cl solution. These findings suggest that the effect of CO_2 removal from perilymphatic perfusion solution on the EP may be mediated by an increase in cytosolic Ca^{2+} concentration induced by an elevation of cytosolic pH in endolymphatic surface cells.

Key words: endocochlear potential, carbon dioxide, intracellular Ca^{2+} , nifedipine, asphyxia.

Pendred's syndrome (PDS), characterized by deafness and goiter, is caused by a mutation of the PDS gene *SLC26A4*, which codes for the protein pendrin [1]. Pendrin, a transporter of HCO_3^- and Cl^- [2, 3], has been found in the endolymphatic surface cells [4]. Furthermore, a positive endocochlear potential (EP), which is essential for the transduction of sound by hair cells, was absent in *Slc26a4*^{-/-} mice [4]. Recently, a review article appeared about the *SLC26* gene family of multifunctional anion exchangers, of which *SLC26A4* is a member [5].

As regards the effects of $\text{CO}_2/\text{HCO}_3^-$ on the generation of the EP, 2 hypotheses could be proposed. The first hypothesis is derived from experiments, showing that the *KCNJ10* K^+ channel was not expressed in intermediate cells in *Slc26a4*^{-/-} mice and that the EP was lost in these animals [6]. These findings suggest that the K^+ diffusion potential across the inner membrane of the intermediate cells, mediated mainly by the *KCNJ10* protein, is the main source of the positive EP [7, 8] and that alterations in the cytosolic pH (pH_c) of intermediate cells in conjunction with free radical stress may lead to the loss of *KCNJ10* protein expression in mice lacking pendrin [6]. The second hypothesis is that the intracellular pH has a strong in-

fluence on the level of the EP, as evidenced by the experiments of Arakawa *et al.* [9], who used the vascular perfusion technique to change the CO_2 level. The results of such experiments suggest that intracellular (marginal cell) alkalization might induce a decrease in the EP. Furthermore, the above investigators proposed that the effect of CO_2 on the EP could also be caused by a change in the level of intracellular Ca^{2+} produced by the change in pH_c , as has been suggested by Roos and Boron [10]. Although it has been considered that $\text{CO}_2/\text{HCO}_3^-$ plays an important role in the generation of the positive EP, no experimental proof has been forthcoming over these last 20 years.

Recent studies have demonstrated that the anoxia- or hypoxia-induced increase in the cytosolic Ca^{2+} concentration ($[\text{Ca}]_c$) in optic nerve or pulmonary arterial smooth muscle cells was inhibited in the presence of an L-type Ca^{2+} channel blocker, diltiazem or nifedipine [11, 12]. Furthermore, studies from our laboratory demonstrated that transient asphyxia induced a large increase in the Ca^{2+} concentration of the endolymph ($[\text{Ca}]_e$) from $\sim 10^{-6}$ to $\sim 10^{-3}$ M and that endolymphatic administration of ethylene-glycol-bis (β -aminoethyl)-*N,N,N',N'*-tetraacetoxymethyl ester (EGTA-AM) caused a slight increase in the rest-

Received on Oct 11, 2006; accepted on Dec 6, 2006; released online on Dec 15, 2006; doi:10.2170/physiolsci.RP012006

Correspondence should be addressed to: Takahiro Kubota, Department of Physiology, Osaka Medical College, 2-7, Daigakumachi, Takatsuki, Osaka, 569-8686 Japan. Tel: +81-726-84- 6421, Fax: +81-726-84-6522, E-mail: ph2008@art.osaka-med.ac.jp

ing EP and inhibited the transient asphyxia-induced decrease in EP (TAID), suggesting that the $[Ca]_c$ in endolymphatic surface cells (ESC) may regulate the generation and maintenance of a positive EP [13, 14]. From all of the above findings, we speculated that the decrease in EP induced by the increase in pH_c in the ESC might have been caused by the increase in $[Ca]_c$ in these cells.

By using ion-selective and conventional microelectrodes and the perilymphatic or endolymphatic perfusion technique, we, therefore, examined whether the effect of CO_2/HCO_3^- on the EP in guinea pigs is caused by the increase in $[Ca]_c$ induced by the increase in pH_c in ESC. A preliminary account of some of our data has been published in abstract form [15].

METHODS

Fabrication and calibration of ion-selective microelectrodes. H^+ -selective microelectrodes were fabricated from silanized pipettes that had been acid-washed and filled with an ion-exchanger (#15251, Fluka). The inner solution was phosphate buffer, pH 7.0, containing 0.1 M NaCl [16]. These microelectrodes silanized with tri-*n*-methylchlorosilane (#41720, Fluka) at 200°C were calibrated between the range of pH from 5.91 to 8.04 with phosphate buffer. The time required to reach 90% of the final voltage in response to the test solutions was less than 1 s for these H^+ -selective microelectrodes. The electric resistance of our microelectrodes was about $2 \times 10^{10} \Omega$. All of the H^+ -selective microelectrodes were calibrated before and after experiments.

Animal preparation and recording system. Guinea pigs with a normal Preyer reflex and weighing between 300 and 400 g were anesthetized by an intraperitoneal injection of pentobarbital sodium (28 mg/kg) and were artificially respirated with room air through a tracheal cannula after the intramuscular injection of suxamethonium chloride (50 mg/kg) [13, 14]. Their ECG was monitored to check the circulatory condition. When we measured the changes in the endolymph (pH_c), the tympanic bulla was exposed; and then a H^+ -selective microelectrode and a conventional microelectrode filled with 0.5 M KCl were inserted into the scala media of the 2nd turn. To measure only the changes in the EP, we inserted the conventional microelectrode into the endolymph. Transient asphyxia was induced by interruption of the artificial ventilation. During the transient asphyxia (~100 s), the heart rate decreased from ~200/min to ~20/min. Therefore, the changes in EP induced by a transient asphyxia were always associated with a decrease in blood perfusion of the cochlea. Tubocurarine (4 μ M) was added to the animal to eliminate neurally mediated twitching of skeletal muscles and associated tissue movement. At the completion of all experimental protocols, animals were given a lethal dose of pentobarbital sodium (i.v.).

As we used a single-barreled H^+ -selective microelectrode in this study, we assured the proper localization of the H^+ -selective microelectrode tip by confirming the abrupt change in voltage in the positive direction to more than 70 mV when the microelectrode was advanced from the cochlear outer surface into the endolymph. The endolymphatic perfusion with solutions containing nifedipine was conducted for more than 5 min while keeping the rate at 80–120 nl/min. The perfusion solution was composed of 125 mM KCl, 25 mM $KHCO_3$, 10^{-7} M Ca^{2+} (adjusted by 1 mM EGTA), and 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and bubbled with 5% CO_2 , 5% O_2 , and 90% N_2 mixed gas. When the solution having about 100 mmHg PO_2 was perfused perilymphatically, the EP was increased and the TAID was suppressed significantly (data are not shown). As the PO_2 of endolymph and perilymph was reported to be 20–30 mmHg [17], we used the perfusion solution bubbled with 5% O_2 gas (equivalent to ~38 mmHg) throughout the current study. The above perfusion solution containing 1 μ g/ml nifedipine was used for perfusion in some experiments. In this study, the double-barreled pipette in which one barrel was used for the measurement of EP and the other for the perfusion was ground by a grinder (EG-1, Narishige, Tokyo) to have a tip diameter of 8–10 μ m.

Perilymphatic perfusion was conducted by use of a pipette having a tip diameter of 30–40 μ m; and the perfusion solution (125 mM NaCl, 25 mM $NaHCO_3$, 1 mM $CaCl_2$ and 5 mM HEPES bubbled with 5% CO_2 , 5% O_2 , 90% N_2) was delivered at a flow rate of 100 μ l/min. A solution containing 10 μ g/ml nifedipine was perfused for more than 10 min via the perilymph at a flow rate of 100 μ l/min or via a catheterized vertebral artery for about 30 s at a flow rate of 1 ml/min.

Each barrel of the microelectrodes used for the measurement of EP and pH_c was connected to a differential electrometer (FD-223, WPI, Sarasota, FL, USA) via a microelectrode holder (MEH1SF, WPI), and the output was recorded by a four-channel recorder (U-638, Nippon Den-shi Kagaku, Tokyo, Japan) and a MacLab 8s (ADInstruments, New South Wales, Australia). The potential from the H^+ -selective microelectrode was recorded by subtracting the potential from the conventional microelectrode for measurement of EP with the differential electrometer. The indifferent electrode was an Ag/AgCl wire connected via an agar bridge containing 3 M KCl to a saline-soaked cotton wick placed on the exposed neck muscles. The rectal temperature of the animals was kept at 37°C by using a DC heating pad. The results were expressed as the mean $\pm$ SD. Statistical significance was addressed by ANOVA's test. *p* values of <0.05 were considered to be significant.

The care and use of animals in this study were approved by the Animal Care and Use Committee of Osaka Medical College.

RESULTS

Effect of perilymphatic perfusion with CO₂/HCO₃⁻-free solution on EP and pH_e

In the first, we assured that both the resting EP and the TAID were not significantly changed by the perilymphatic perfusion with an artificial solution of 5% CO₂/25 mM HCO₃⁻ (5% O₂). These results are shown in Fig. 1 and summarized in Table 1.

A typical time course of the change in the EP ($\Delta EP_{CO_2\text{-free}}$) and pH_e induced by the perilymphatic perfusion with CO₂/HCO₃⁻-free solution is shown in Fig. 2. The EP was decreased from +80 to +52 mV with a large increase in pH_e (from 7.45 to 7.87) during a 10-min perilymphatic perfusion with CO₂/HCO₃⁻-free solution, indicating that lowering the CO₂/HCO₃⁻ level in the perilymph induced a decrease in the EP. By contrast, the change in the EP induced by the transient asphyxia (ΔEP_{asp}) during 100 s was ~48 mV (from +83 to +35) with a slight decrease in pH_e (ΔpH_e , ~0.05) during the perilymphatic perfusion with the solution containing 5% CO₂/25 mM HCO₃⁻ (Fig. 2). The change in EP induced by the perilymphatic perfusion with CO₂/HCO₃⁻-free solution (10 min) is given in Table 1. However, when the perfusion with the CO₂/HCO₃⁻-free solution was done for >20 min, ~15 min was needed to reach a new steady state EP after the start of perfusion, as shown in Fig. 3.

Effect of perilymphatic perfusion with high or low HCO₃⁻ solution on EP

In the next experiment, we examined which of the changes, CO₂ or HCO₃⁻, in the perilymph caused the

Table 1. Effect of CO₂/HCO₃⁻ on EP and pH_e during the perilymphatic perfusion with 25 mM or 0 mM HCO₃⁻ at 5% or 0% CO₂ level. The values were obtained at 10 min after perfusion with each solution.

	Control	5% CO ₂ 25 mM HCO ₃ ⁻	0% CO ₂ HEPES
EP (mV)	77.6 ± 4.6 (n = 21)	75.6 ± 6.2 ^{ns} (n = 5)	48.4 ± 6.1* (n = 5)
pH _e	7.45 ± 0.03 (n = 17)	7.45 ± 0.02 ^{ns} (n = 5)	7.86 ± 0.04* (n = 5)

Data are presented as the mean ± SD. *n*, number of observations. ^{ns}, Not significantly different from the control value. *, Significantly different from the value obtained after the perfusion with 5% CO₂ and 25 mM HCO₃⁻ solution (*p* < 0.0001).

Fig. 2. Changes in EP and pH_e in response to 10-min perilymphatic perfusion with CO₂/HCO₃⁻-free solution. The EP was decreased by perfusion with CO₂/HCO₃⁻-free solution concomitant with a large increase in pH_e. Transient asphyxia during perfusion with 5% CO₂/25 mM HCO₃⁻-containing solution produced a large decrease in EP, whereas it produced only a slight decrease in pH_e.

Fig. 1. Effect of perilymphatic perfusion with artificial solution (5% CO₂/25 mM HCO₃⁻) on EP. Neither resting EP nor TAID were significantly affected by perilymphatic perfusion with the artificial solution (5% CO₂/25 mM HCO₃⁻).

Fig. 3. Changes in EP in response to perilymphatic perfusion with CO₂/HCO₃⁻-free solution for more than 20 min. The EP decreased to +25 mV and reached a new steady state potential; and TAID was suppressed (peak of EP at 1.5 min of transient asphyxia was lowered).

Table 2. Effect of $\text{CO}_2/\text{HCO}_3^-$ on EP and pH_e during the perilymphatic perfusion with different HCO_3^- concentrations at the 5% CO_2 level. The values were obtained at 10 min after perfusion with each solution.

Perilymphatic perfusion solution			
pH	7.47	6.77	7.77
HCO_3^-	25 mM	5 mM	50 mM
EP (mV)	77.2 ± 3.4 ($n = 17$)	$76.6 \pm 3.6^{\text{ns}}$ ($n = 5$)	$76.2 \pm 3.0^{\text{ns}}$ ($n = 5$)
pH_e	7.45 ± 0.03 ($n = 16$)	$7.36 \pm 0.04^*$ ($n = 5$)	$7.53 \pm 0.03^{**}$ ($n = 5$)

Data are presented as the mean $\pm$ SD. n , number of observations. ^{ns}, Not significantly different from the value for perilymphatic perfusion with 25 mM HCO_3^- solution.

^{*}, ^{**}Significantly different from the value for perilymphatic perfusion with 25 mM HCO_3^- solution at level of $p < 0.005$ or $p < 0.001$. The values of EP and pH_e during the perilymphatic perfusion with 25 mM HCO_3^- solution were not significantly different from the control value in Table 1.

$\Delta\text{EP}_{\text{CO}_2\text{-free}}$. Lowering the HCO_3^- concentration and pH with a constant CO_2 (5%) also produced no significant change in EP with a slight decrease in pH_e (ΔpH_e , ~ 0.1), as summarized in Table 2. As seen in Table 2, elevating the HCO_3^- concentration and pH while maintaining a constant CO_2 (5%) level in the perilymph produced no significant change in the EP 10 min after the initiation of the perfusion of experimental solution, though there was a slight increase in pH_e (ΔpH_e , ~ 0.1). However, the EP decreased slightly (~ 4 mV) with the change in pH_e (ΔpH_e , ~ 0.3) 30 min after the perfusion with 50 mM HCO_3^- containing solution (data are not shown). These results are similar to those of previous experiments using the vascular perfusion technique, which indicated that pH_e had a strong influence but that the extracellular pH had no effect on the level of the EP [9]. Therefore, we suspected that decreasing the perilymphatic CO_2 , but not HCO_3^- , may have caused alkalization in the perilymphatic perfusion area (or ESC), resulting in the $\text{CO}_2/\text{HCO}_3^-$ -free-induced decrease in the EP. To test this possibility, we performed perilymphatic perfusion with a solution containing 20 mM NH_4Cl .

Effects of perilymphatic perfusion with NH_4Cl on EP and pH_e

It is well known that intracellular alkalization is induced by the application of NH_4Cl and may be reversed by the washout of NH_4Cl [10]. So next we examined whether the reduction in EP during the perilymphatic perfusion with $\text{CO}_2/\text{HCO}_3^-$ -free solution was due to the increase in the pH_e of the cells in the perilymphatic perfusion area. As shown by individual tracing in Fig. 4, a reduction in EP associated with an increase in pH_e was observed by the perilymphatic perfusion with 20 mM NH_4Cl . Recovery of the EP and pH_e was observed after the washout of the NH_4Cl , although transient decrease in the pH_e was not seen after the washout. The changes in EP ($\Delta\text{EP}_{\text{NH}_4\text{Cl}}$) and pH_e induced by perilymphatic perfusion

Fig. 4. Changes in EP and pH_e in response to perilymphatic perfusion with NH_4Cl solution. The EP was decreased by perilymphatic perfusion with 20 mM NH_4Cl solution with a concomitant large increase in pH_e .

Fig. 5. Effect of transient asphyxia on EP with or without endolymphatic administration of nifedipine. TAID was inhibited by the endolymphatic administration of nifedipine.

with NH_4Cl were 38.5 ± 8.3 mV (mean $\pm$ SD, $n = 5$) and an increase in the pH_e from 7.48 ± 0.06 to 7.62 ± 0.06 (mean $\pm$ SD, $n = 5$), respectively, after the 5 min perfusion. When the perilymph was perfused with the NH_4Cl -containing solution for more than 10 min, no recovery of the EP was seen, suggesting that this high NH_4Cl solution was toxic to the stria vascularis (data are not shown). It is reasonable, however, to speculate that the pH_e in the cells of perilymphatic perfusion area would change in the same direction as the pH_e during the perilymphatic perfusion with NH_4Cl . Therefore, these results suggest that the increase in the pH_e of ESC may have produced the $\text{CO}_2/\text{HCO}_3^-$ -free-induced decrease in EP.

As shown in Fig. 5, endolymphatic administration of nifedipine suppressed significantly the TAID, indicating that the increase in $[\text{Ca}]_c$ induced by Ca^{2+} -influx into ESC

Fig. 6. Change in EP in response to perilymphatic perfusion with CO₂/HCO₃⁻-free solution (A) or NH₄Cl solution (B) with or without endolymphatic application of nifedipine. A solution of 5% CO₂ (25 mM HCO₃⁻) solution was perfused perilymphatically throughout the experiments except when CO₂/HCO₃⁻-free solution was perfused. (A) ΔEP_{asp} and $\Delta EP_{CO_2\text{-free}}$ were significantly lowered by application of 1 µg/ml nifedipine. (B) ΔEP_{NH_4Cl} was also significantly less by application of 1 µg/ml nifedipine.

through nifedipine-sensitive Ca²⁺ channel may regulate the EP. Therefore, we also examined the effect of nifedipine on the reduction in EP induced by the perilymphatic perfusion with CO₂/HCO₃⁻-free or NH₄Cl solution to see whether such an influx of Ca²⁺ was a part of the mechanism responsible for the reduction in the EP.

Endolymphatic or perilymphatic perfusion with nifedipine

It was reported that nifedipine, a lipid soluble Ca²⁺ channel blocker, inhibited the acute hypoxia-induced increase in [Ca]_c of pulmonary arterial smooth muscle [12].

Table 3. Changes in EP induced by perilymphatic perfusion with CO₂/HCO₃⁻-free ($\Delta EP_{CO_2\text{-free}}$), or NH₄Cl (ΔEP_{NH_4Cl}) with or without nifedipine administration via the endolymph.

	$\Delta EP_{CO_2\text{-free}}$ (after 10 min)	ΔEP_{NH_4Cl} (after 5 min)
(control)	35.0 ± 5.5 mV (n = 5)	45.8 ± 5.5 mV (n = 5)
+ nifedipine (endolymph)	12.5 ± 5.5 mV* (n = 5)	15.3 ± 5.6 mV* (n = 5)

Data are presented as the mean ± SD. *n*, number of observations. *Significantly different from the control value (*p* < 0.001).

In the current study, therefore, we also examined the effect of nifedipine on the reduction in EP induced by the transient asphyxia, or by the perilymphatic perfusion with CO₂/HCO₃⁻-free or NH₄Cl solution. As shown in Fig. 6A, the endolymphatic application of 1 µg/ml nifedipine significantly suppressed the reduction in EP induced by the transient asphyxia or the perilymphatic perfusion with CO₂/HCO₃⁻-free solution. Furthermore, the NH₄Cl-induced decrease in the EP was inhibited significantly by the endolymphatic administration of 1 µg/ml nifedipine, shown in Fig. 6B. All of these results are summarized in Table 3. By contrast, perilymphatic administration of 10 µg/ml nifedipine produced no significant inhibition of the ΔEP_{asp} , as shown in Fig. 7.

Finally, we examined the effect of nifedipine on the TAID by delivering it via a vertebral artery, because the stria vascularis is supplied by the blood flow through a branch of this artery. The application of nifedipine into a vertebral artery strongly lowered ΔEP_{asp} (45.5 ± 8.3 mV without nifedipine, 12.5 ± 8.3 mV after Ringer injections with nifedipine, *p* < 0.001), as shown in Fig. 8. From these results, we consider it possible that prevention of Ca²⁺-influx into ESC through nifedipine-sensitive Ca²⁺ channel

Fig. 7. Effect of transient asphyxia on EP with or without perilymphatic administration of nifedipine. TAID was not inhibited by the endolymphatic administration of nifedipine and EP was gradually decreased.

Fig. 8. Effect of nifedipine via vertebral artery on the changes in EP induced by transient asphyxia. The decrease in EP induced by transient asphyxia was inhibited by the administration of nifedipine via vertebral artery, although gradual decrease in EP was observed.

contributes to the inhibitory mechanism of the reduction in EP induced by the perilymphatic perfusion with $\text{CO}_2/\text{HCO}_3^-$ -free or NH_4Cl solution and that this potential may be generated or maintained by the cells around vessels and near the endolymph, but not by the basal cells.

DISCUSSION

The present study demonstrates that (i) the perilymphatic perfusion with $\text{CO}_2/\text{HCO}_3^-$ -free solution produced a decrease in the EP, whereas the perfusion with a solution of high or low HCO_3^- with a constant CO_2 level produced no significant changes in the EP; (ii) that the perilymphatic perfusion with an NH_4Cl -containing solution produced a decrease in the EP; and (iii) that nifedipine inhibited the reduction in EP induced by transient asphyxia or by the perilymphatic perfusion with $\text{CO}_2/\text{HCO}_3^-$ -free or NH_4Cl solution, probably by blocking the increase in $[\text{Ca}]_c$ in ESC. These results indicate that reducing the CO_2 level in the perilymph ($\text{CO}_2/\text{HCO}_3^-$ -free perfusion) elevated the pH_c and therefore might have increased the pH_c in the ESC, which increase, in turn, may have induced an increase in $[\text{Ca}]_c$ of the ESC that have been caused by the activating nifedipine-sensitive Ca^{2+} channel.

Effect of CO_2 in perilymph on the EP

Based on the results of a study using a vascular perfusion technique to change the CO_2 level, Arakawa *et al.* [9] suggested that the pH_c of EP-generating cells had a strong influence on the level of the EP. In general, increasing extracellular CO_2 causes a decrease in pH_c and decreasing

extracellular CO_2 has the reverse effect on it, especially in muscle fibers [18]. Presume that the cation composition of the endolymph is almost the same as that of the cytosol of the ESC [19], we propose that the increase in the pH_c of ESC was produced by a low CO_2 level in the perilymph during the perfusion with the $\text{CO}_2/\text{HCO}_3^-$ -free solution, resulting in a decreased EP. The results showing the decrease in EP induced by perilymphatic perfusion with NH_4Cl may support the above speculation, because brief NH_3/NH_4 exposure has been shown to cause an increase in pH_c [10, 20]. By contrast, in the present study the perilymphatic perfusion with high or low HCO_3^- at a constant CO_2 level, which is known to have no significant effect on pH_c [9], gave no significant effects on the EP. From these experimental results, we conclude that an increase in the pH_c of ESC, induced by a decrease in extracellular CO_2 , would produce a decrease in the EP, as the basal cells are not very permeable to H^+ or HCO_3^- .

On the other hand, in renal proximal tubule cells, which possess a powerful H^+ -extrusion system, such as H^+ -ATPase and Na^+/H^+ exchanger, and abundant carbonic anhydrase, an increase in pH_c instead of a decrease in it is produced by the exposure to high extracellular CO_2 [18, 21, 22]; although the initial pH_c is an important factor [23]. In cytochemical and histochemical studies, abundant carbonic anhydrase and H^+ -ATPase have been demonstrated in endolymphatic surface cells of the stria vascularis in guinea pigs [24–26]. In light of these findings, we also examined the effect of high CO_2 in the perilymph on the changes in EP and pH_c . Elevating CO_2 from 5% to 15% in the perilymph produced a slight decrease in the pH_c without a significant change in the EP (pH_c : from 7.48 ± 0.05 to 7.38 ± 0.05 , EP: from $+75.2 \pm 6.5$ to 76.3 ± 5.8 mV; unpublished observation). Therefore, it is not likely that the pH_c in ESC was affected significantly by an elevation of the CO_2 level in the perilymph as long as the elevation was from 5% to 15%. Thus, we consider that the effect of both the $\text{CO}_2/\text{HCO}_3^-$ -free and NH_4Cl solutions on the EP resulted from intracellular alkalinization in the ESC.

In addition to the effect of CO_2 on the EP, HCO_3^- transport in the stria vascularis must be considered; although the changing the HCO_3^- and H^+ levels at a constant CO_2 in the perilymph caused no significant change in the EP in the current study. In $\text{SLC26}^{-/-}$ mice, deafness and loss of EP were observed with loss of KCNJ10 expression in the intermediate cells [6]. The mechanism of this loss in expression in intermediate cells in $\text{SLC26}^{-/-}$ mice is still unknown, but it was speculated that the loss of pendrin, which may transport HCO_3^- into the endolymph, results in an accumulation of HCO_3^- and an alkalinization of the intrastrial spaces in the stria vascularis. This extracellular alkalinization may enhance free radical stress on the intermediate cells, since it may inhibit the uptake of cysteine into these cells and thereby limit the production of the pro-

protective glutathione [6]. Apart from the discussion about free radical stress, it is conceivable that an alkalization of the intrastrial spaces might be produced, resulting the alkalization of ESC in the absence of pendrin. If this is in the case, this increase in pH_c in these cells might cause the decrease in the EP as shown in the current study. In any case, the pH_c of ESC including the pH of the intrastrial spaces may play an important role in the regulation of the EP.

Perilymphatic perfusion with CO₂/HCO₃⁻-free or NH₄Cl solution might produce an increase in [Ca]_c of ESC

As was shown in Figs. 2 and 4, the EP was decreased gradually with a large increase in pH_c caused by the perilymphatic perfusion with CO₂/HCO₃⁻-free or NH₄Cl solution, whereas it was largely decreased with a slight change in the pH_c by the transient asphyxia. This result indicates that TAID was not caused by a change in the pH_c of ESC or in the pH of the extracellular space surrounding these cells. It has been reported that an increase in [Ca]_c was induced by the transient asphyxia [13] and that TAID was inhibited by the endolymphatic administration of EGTA-AM [14]. Furthermore, the reduction in EP induced by transient asphyxia or the perilymphatic perfusion with CO₂/HCO₃⁻-free or NH₄Cl solution was inhibited by the endolymphatic or intravascular administration of nifedipine in our current study. Therefore, we considered that a part of the reduction in EP induced by the perilymphatic perfusion with CO₂/HCO₃⁻-free or NH₄Cl solution might have been caused by the increase in [Ca]_c of ESC.

A relationship between [Ca]_c and pH_c was demonstrated in renal proximal tubule cells [21, 27], which have the similar epithelial function as ESC. By contrast, it was demonstrated that no correlation was observed between [Ca]_c and pH_c in the rabbit proximal tubule cells [23]. At the present time, therefore, one should be careful to conclude that increasing the pH_c causes an increase in the [Ca]_c and that decreasing it produces the reverse effect on the cells. As demonstrated presently, however, nifedipine inhibited the reduction in EP induced by the perilymphatic perfusion with either CO₂/HCO₃⁻-free or NH₄Cl solution. Thus, we hypothesize that lowering the CO₂ level in the perilymph resulted in an increase in pH_c to produce the increase in [Ca]_c in ESC. Although it was reported that an increase in pH_c activates L-type Ca²⁺ channel in vascular smooth muscle, the molecular basis of pH_c regulation of L-type Ca²⁺ channel is still unclear [28]. In any case, no firm conclusion should be made until the pH_c and [Ca]_c of ESC are measured in future experiments.

Hypoxia or anoxia-induced increase in [Ca]_c

It has been reported that hypoxia or anoxia increases [Ca]_c in various tissues, including those of the central ner-

vous system [11], as well as in arterial smooth muscle cells [12] and *Drosophila* retinal cells [13]. This increase in [Ca]_c induced by anoxia is caused by the Ca²⁺ influx through the activation of L-type Ca²⁺ channel [11, 12] or TRP channels [29]. In the studies just cited, channel blockers such as nifedipine, diltiazem, or La³⁺ inhibited the increase in [Ca]_c induced by anoxia. Furthermore, Mineharu et al. reported that TAID in was inhibited by endolymphatic administration of EGTA-AM but not by perilymphatic administration of this chelator [14]. The inhibitory effect of nifedipine on TAID in the current study (Fig. 5) was almost the same as that of the endolymphatic administration of EGTA-AM (Fig. 6 in Ref. [14]). Furthermore, endolymphatic administration of nifedipine increased the basal level of the EP (Figs. 5 and 6) as seen in the endolymphatic administration of EGTA-AM (Fig. 8 in Ref. [14]). Therefore, we consider that nifedipine inhibits L-type Ca²⁺ channels in ESC, although it has been reported that nifedipine also inhibits non-specifically several types of ion channels or transporters, such as Kv4.2, Kv4.3, Kv1.4 or P-glycoprotein [30, 31].

In the current study, we demonstrated that administration of nifedipine into the endolymph or via a vertebral artery inhibited significantly TAID. We also examined the effects of perilymphatic administration of La³⁺ or Cd²⁺ on the EP, but could not detect any significant inhibition of the TAID (data are not shown). Therefore, we propose that (i) anoxia- or asphyxia-activated L-type Ca²⁺ channel participated in the increase in [Ca]_c of ESC and the decrease in the EP, and (ii) vessel-surrounding cells in the stria vascularis, such as marginal cells and intermediate cells, may contribute to the generation of the EP.

In conclusion, an increase in the pH_c of ESC, as well as pH of the intrastrial spaces, may produce an increase in [Ca]_c through the activation of L-type Ca²⁺ channel, resulting in the decrease in EP. Further experiments are needed to clarify the role of Ca²⁺ in the regulation of the large positive EP.

REFERENCES

1. Everett LA, Glaser B, Beck JC, Idol JR, Buchs A, Heyman M, Adawi F, Hazani E, Nassir E, Baxavanis AD, Sheffield VC, Green ED. Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS). *Nat Genet.* 1997;17:411-22.
2. Scott DA, Wang R, Kreman TM, Sheffield VC, Karniski LP. The Pendred syndrome gene encodes a chloride-iodide transport protein. *Nat Genet.* 1999;21:440-3.
3. Scott DA, Karniski LP. Human pendrin expressed in *Xenopus laevis* oocytes mediates chloride/formate exchange. *Am J Physiol.* 2000;278:C207-11.
4. Royaux IE, Belyantseva IA, Wu T, Kachar B, Everett LA, Marcus DC, Green ED. Localization and functional studies of pendrin in the mouse inner ear provide insight about the etiology of deafness in Pendred syndrome. *JARO.* 2003;4:394-404.
5. Mount DB, Romero MF. The SLC26 gene family of multifunctional anion exchangers. *Pflügers Arch.* 2004;447:710-21.
6. Wangemann P, Itza EM, Albrecht B, Jabba SV, Maganti RJ, Lee JH, Everett LA, Wall SM, Royaux IE, Green ED, Marcus DC. Loss of KCNJ10 protein expression abolishes endocochlear potential and causes deafness in Pendred syndrome

- mouse model. *BMC Med.* 2004;2:1-30.
7. Wangemann P, Liu J, Marcus DC. Ion transport mechanisms responsible for K⁺ secretion and the transepithelial voltage across marginal cells of stria vascularis in vitro. *Hear Res.* 1995;84:19-29.
 8. Wangemann P. K⁺ cycling and the endocochlear potential. *Hear Res.* 2002;165:1-9.
 9. Arakawa E, Marcus DC, Thalmann R. Dependence of endocochlear potential on vascular pH. *Hear Res.* 1987;31:1-7.
 10. Roos A, Boron WF. Intracellular pH. *Physiol Rev.* 1981;61:296-434.
 11. Brown AM, Weatenbroek RE, Catterall WA, Ransom BR. Axonal L-type Ca²⁺ channels and anoxic injury in rat CNS white matter. *J. Neurophysiol.* 2001;85:900-11.
 12. Wang J, Shimoda LA, Weigand L, Wang W, Sun D, Sylvester JT. Acute hypoxia increases intracellular [Ca²⁺] in pulmonary arterial smooth muscle by enhancing capacitive Ca²⁺ entry. *Am J Physiol.* 2005;288:L1059-69.
 13. Takamaki A, Mori Y, Araki M, Yamaji J, Yoshida R, Takenaka H, Kubota T. Asphyxia and diuretic-induced changes in the Ca²⁺ concentration of endolymph. *Jpn J Physiol.* 2003;53:35-44.
 14. Mineharu A, Mori Y, Nimura Y, Takamaki A, Araki M, Yamaji J, Yoshida R, Takenaka H, T. Kubota Endolymphatic perfusion with EGTA-acetoxymethyl ester inhibits asphyxia- and furosemide-induced decrease in endocochlear potential in guinea pigs. *Jpn J Physiol.* 2005;55:53-60.
 15. Nimura Y, Mori Y, Yamaji J, Yoshida R, Takenaka H, Kubota T. Effect of CO₂/HCO₃⁻ on endocochlear potential in guinea pigs. *Jpn J Physiol.* 2005;55:S158.
 16. Kubota T, Hagiwara N, Inoue A, Fujimoto M. The effect of ouabain, insulin, and cyclic AMP on the acidification of luminal fluid in the proximal tubule of bullfrog kidney. *Jpn J Physiol.* 1988;38:549-56.
 17. Prazma J. Perilymphatic and endolymphatic PO₂: Variation during anoxia, hypoxia, and hypercapnia. *Arch Otolaryngol* 1982;539-43.
 18. Bevensee MO, Schmitt BM, Choi I, Romero MF, Boron WF. An electrogenic Na⁺-HCO₃⁻ cotransporter (NBC) with a novel COOH-terminus, cloned from rat brain. *Am J Physiol* 2000;278:C1200-11.
 19. Sterkers O, Ferrary E, Amiel C. Production of inner ear fluids. *Phys Rev.* 1988;68:1083-128.
 20. Boron WF. Intracellular pH transients in giant barnacle muscle fibers. *Am J Physiol.* 1977;233:C61-73.
 21. Fujimoto M, Kubota T, Hagiwara N, Ohno-Shosaku T, Kubokawa M, Kotera K. Ion-selective microelectrodes to study proton and bicarbonate transport in the renal epithelium. *Kidney Int.* 1991;33:S23-8.
 22. Nakhoul NL, Chen LK, Boron WF. Effect of basolateral CO₂/HCO₃⁻ on intracellular pH regulation in the rabbit S3 proximal tubule. *J Gen Physiol.* 1993;102:1171-205.
 23. Bouyer P, Zhou Y, Boron WF. An increase in intracellular calcium concentration that is induced by basolateral CO₂ in rabbit renal proximal tubule. *Am J Physiol.* 2003;285:F674-87.
 24. Hsu CJ, Nomura Y. Carbonic anhydrase activity in the inner ear. *Acta Otolaryngol Suppl (Stockh).* 1985;418:1-42.
 25. Okamura H, Sugai N, Suzuki K, Ohtani I. Enzyme-histochemical localization of carbonic anhydrase in the inner ear of the guinea pig and several improvements of the technique. *Histochem Cell Biol.* 1996;106:425-30.
 26. Stankovic KM, Brown D, Alper SL, Adams JC. Localization of pH regulating proteins H⁺ATPase and Cl⁻/HCO₃⁻ exchanger in the guinea pig inner ear. *Hear Res.* 1997;114:21-34.
 27. Fujimoto M, Kubota T, Hagiwara N, Kubokawa M, Ohno-Shosaku T, Kotera K. Relationship between cytosolic activities of calcium and pH in frog proximal tubules. *Jpn J Physiol.* 1990;40:273-96.
 28. Wakabayashi I, Poteser M, Groschner K. Intracellular pH as a determinant of vascular smooth muscle function. *J Vasc Res.* 2006;43, 238-50.
 29. Minke B, Cook B. TRP channel proteins and signal transduction. *Physiol Rev.* 2002;8:429-72.
 30. Hatano H, Ohya S, Murai K, Giles W, Imaizumi Y. Dihydropyridine Ca²⁺ channel antagonists and agonists block Kv4.2, Kv4.3 and Kv1.4 K⁺ channels expressed in HEK293 cells. *Brit J Pharmacol.* 2003;139:533-44.
 31. Harris AL, Hochhauser D. Mechanism of multidrug resistance in cancer treatment. *Acta Oncol.* 1992;31:205-13.