pH$_i$ in Piglet Cerebral Microvascular Endothelial Cells: Recovery from an Acid Load (44014)

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Abstract. **Cerebral microvascular endothelial cells form a barrier between the blood and brain, which is critical for normal neuronal functions. These endothelial cells can be challenged by metabolic and respiratory acidosis, especially in newborn babies. We investigated mechanism(s) by which cerebral endothelial cells recover intracellular pH (pH$_i$) when challenged with an intracellular acid load. pH$_i$ in piglet cerebral microvascular endothelial cells in primary culture was monitored using the pH-sensitive fluorescent dye BCECF (2′,7′-bis-2-carboxyethyl-5(6)-carboxy-fluorescein acetoxymethyl ester), with dual wavelength fluorescence spectroscopy. Endothelial cells attached to coverslips and continuously superfused with HCO_3^-/CO_2 containing medium (25 m*M* HCO_3^-, 5% CO_2; pH 7.40) have a steady state of pH$_i$ of 7.18 ± 0.02. Under basal conditions, amiloride (100 μ*M*) and H_2DIDS (0.5 m*M*) decreased pH$_i$ 0.12 ± 0.01 and 0.05 ± 0.01 pH units, respectively. Removal of external Na^+ lowered pH$_i$ 0.18 ± 0.02 pH units, while Cl^--free medium decreased pH$_i$ 0.16 ± 0.03 pH units. These data suggest the presence of an amiloride-sensitive Na^+-H^+ exchanger and a Na^+-dependent HCO_3^--Cl^- anion exchanger in endothelial cells. Propionate and high PCO_2 cause rapid intracellular acidification at constant pH$_e$. The cells recover to control pH$_i$ over 10 min. Recovery from propionate was largely inhibited by amiloride, slightly inhibited by H_2DIDS, and completely prevented by the combination. pH$_i$ recovery during elevated PCO_2 was blocked by amiloride, H_2DIDS, or Na^+-free media. These results indicate that recovery from intracellular acidosis can involve amiloride-sensitive Na^+-H^+ exchange and a Na^+-dependent HCO_3^-/Cl^- anion exchange. Relative contributions of pumps and their independence appears to depend on the nature of the acid load.**

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The endothelium is a confluent monolayer of thin, flattened, rhomboid-shaped cells lining the intimal surface of all blood vessels that plays an important role in physiologic homeostasis (1). Brain microvascular endothelial cells also form the blood-brain barrier (BBB), which contributes to the stability of the brain parenchymal microenvironment. Defects in brain microvascular endothelial cells may result in breakdown of the BBB and produce brain injury. Hence, the normal function and structure of brain microvascular endothelial cells are critical to normal brain function.

Intracellular pH (pH$_i$) is a prominent determinant of the rate of many intracellular processes and has been implicated in the regulation of vital cellular functions. Many biological compounds such as proteins have acid or base groups, and the degree of their protonation is critical for their biological functions (2–4). The cerebral endothelial cells might be challenged by respiratory or metabolic acidosis, especially during the

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newborn period. Acid-extruding mechanisms are important for the maintenance of a steady pH_i in tissue. pH_i is regulated by several routes, including intracellular physiochemical buffers, a Na^+-H^+ exchanger, Na^+-dependent and independent HCO_3^--Cl^- exchangers, a Na^+-HCO_3^- cotransporter, passive leakage, and H^+-ATPase (5, 6).

Previously, we demonstrated that pH_i in endothelial cells could be decreased by weak acids, decreased pH_e, and high CO_2. pH_i was decreased initially, but recovered exponentially to its basal values if pH_e was maintained normal at 7.4 but not if pH_e also fell, indicating that piglet cerebral microvascular endothelial cells require a helping pH gradient to recover from intracellular acidosis (7). The membrane channels and pumps allowing such recovery from an intracellular acid load in these cells have not been established. The current study, therefore, was designed to investigate the pH_i homeostatic mechanisms operating in response to an acid load. Specifically, we have examined the influence of amiloride, H_2DIDS, Na^+, and Cl^- on basal pH_i and pH_i recovery after intracellular acidification by a weak acid or CO_2 under a HCO_3^-/CO_2 buffered condition.

Materials and Methods

All procedures involving animals were reviewed and approved by the Animal Care and Use Committee at the University of Tennessee, Memphis. All procedures were done using sterile techniques.

Chemicals and supplies. BCECF/AM (fluorescent probe 2′,7′-bis-2-carboxyethyl-5(6)-carboxyfluorescein acetoxymethyl ester) and H_2DIDS (dihydro-4′,4′-diisothiocyanostilbene-2,2′-disulfonic acid) were purchased from Molecular Probes (Eugene, OR). Leighton tissue culture tubes and glass coverslips were from Bellco Biotechnology (Vineland, NJ). Nylon Mesh Screen was obtained from Spectrum (Houston, TX). Percoll and density markers were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Collagenase/dispase was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Fetal bovine serum was purchased from HyClone (Logan, UT). All other chemicals or biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation and culture of microvascular endothelial cells. Piglet cerebral microvascular endothelial cells were isolated by mechanical dispersion and collagenase/dispase digestion as previously described (7, 8). Briefly, brain cortex from piglets 1–3 days of age was removed under anesthesia (ketamine/acepromazine [33/3.3 mg/kg]), placed into a beaker with 40 ml cold isolation solution containing tissue culture Medium 199, 0.015 *M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 1 U/ml sodium heparin, antibiotic-antimycotic solution (100 U/ml penicillin, 100 μg/ml steptomycin, and 2.5 μg/ml amphotericin B), washed three times with isolation solution, minced into tiny pieces using two scalpels in 20 ml isolation solution, and then transferred to a 40-ml Dounce homogenizer and homogenized with 10 strokes of a loose-fitting pestle. The homogenate was passed through 300- and 60-μm nylon mesh screens. The 60-μm nylon mesh screen was removed and placed in a 50-ml centrifuge tube containing 50 ml of isolation solution. The microvessels (60–300 μm) were washed off by agitation and scraping, then centrifuged at 500*g* for 5 min. The isolated microvessels were suspended in 10 ml of collagenase/dispase at 1 mg/ml in isolation solution and agitated 3 hr on a gyratory shaker (100 rpm) at 37°C to separate endothelial cells from glia and smooth muscle cells. Percoll gradients containing 50% Percoll were established by centrifugation at 26,000*g* for 1 hr in 50-ml polycarbonate tubes in an angle rotor. The density of Percoll was determined with commercially available density markers. Cells in isolation solution (2 ml), which were collected by centrifugation after enzyme treatment, were layered over the (36 ml) 50% Percoll gradients and banded by centrifugation in a swinging bucket rotor at 1000*g* for 10 min. The endothelial band (Band 2, at a density between 1.052 and 1.055 g/ml was removed with a syringe and 16-gauge needle, passed through a 26-gauge needle, diluted with 30 ml of isolation solution, and collected by centrifugation at 1000*g* for 8 min.

The pellet was resuspended using preincubated culture medium consisting of Dulbecco's Modified Eagle's Media (DMEM[E]) containing 20% fetal bovine serum (FBS), 2 mg/ml sodium bicarbonate, 1 U/ml sodium heparin, 30 μg/ml endothelial cell growth supplement, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. The cell suspensions were seeded onto 9 × 35-mm Matrigel-coated glass coverslips within Leighton tissue culture tubes (16 × 93 mm) and were grown to confluence in DMEM(E) under 5% CO_2 in air at 37°C.

Measurement of intracellular pH. pH_i of adherent endothelial cells was measured using the pH-sensitive fluorescent dye BCECF (9). Cells attached to coverslips were incubated for 10 min at 37°C in 1 ml aCSF (150 Na^+ mEq/l, 3 K^+ mEq/l, 2.5 Ca^{2+} mEq/l, 1.2 Mg^{2+} mEq/l, 132 Cl^- mEq/l, 3.7 m*M* glucose, 6 m*M* urea, 25 HCO_3^- mEq/l; pH 7.40; PCO_2 35) containing 2.9 μ*M* BCECF acetoxymethyl ester. Stock solutions of 1.45 m*M* BCECF/AM were made in dimethyl sulfoxide (DMSO) and could be stored at −20°C for 3 months. For experimentation, BCECF stock was thawed and added to loading media. The coverslip containing the cells was then placed into a 1-ml cuvette, contained in an LS-50 spectrofluorometer (Perkin-Elmer, Norwalk, CT) fitted with a water-jacketed cuvette holder that maintained the cells at

37°C. The coverslip was positioned in the spectrofluorometer at a 45° angle to the excitement beam. Cells then superfused at a rate of 3 ml/min with aCSF. Measurement of the fluorescence intensity of BCECF was performed with excitation wavelength pairs of 505/439 nm (slit, 5 nm) and an emission wavelength of 530 nm (slit, 10 nm). After a constant fluorescence ratio was observed, cells were superfused for an additional 5 min with aCSF to obtain basal values for pH_i. The medium was then changed to an experimental medium for another 15 min.

The ratio of fluorescence at 530 nm was corrected for autofluorescence (measured for each coverslip before loading BCECF). Calibration of the fluorescence ratio to pH_i was performed on each coverslip after the experiment by using the monovalent cation ionophore nigericin to equilibrate pH_i with extracellular pH (pH_e) when intracellular $[K^+]$ equaled extracellular $[K^+]$ (10). The calibration solution contained 145 m*M* KCl, 22 m*M* HEPES, 5 μ*M* nigericin, and all other chemicals in aCSF except NaCl and $NaHCO_3$, and was titrated to pH_e values between 6.5 and 7.5 by using 5 *N* KOH. The correlation of the ratio of fluorescence intensity (506/455) and pH was then analyzed by linear regression to calculate pH_i using fluorescent data management software developed by Perkin-Elmer.

Statistical analysis. Results are expressed as means ± SEM of *n* preparations. Statistical analysis was performed using analysis of variance and a *post hoc* Fisher protected least significant difference test. Values of $P < 0.05$ were regarded as significant. Data presented in the text as changes are different by $P < 0.05$.

Results

Effect of amiloride, H_2DIDS, external Na^+, and Cl^- on pH_i The mean pH_i of piglet cerebral microvascular endothelial cells was 7.18 ± 0.02 ($n = 60$) in aCSF ($pH_e = 7.4$) equilibrated with 5% CO_2, 21% O_2, and 74% N_2 at 37°C. Application of amiloride (100 μ*M*) produced an acidification of 0.12 ± 0.01 pH units ($n = 5$) in piglet cerebral microvascular endothelial cells perfused in control aCSF (Fig. 1A). A higher concentration of amiloride (1 m*M*) did not decrease pH_i more (data not shown). Since literature data suggest 100 μ*M* should be adequate (11, 13) and a higher concentration had no greater effect, only 100 μ*M* amiloride was used in these experiments. Addition of H_2DIDS (0.5 m*M*) resulted in a pH_i drop of 0.05 ± 0.01 pH units ($n = 10$) Fig. 1B). No higher concentrations of H_2DIDS were used because this is the maximal concentration used in most reports (11, 13) and this dose totally blocked pH_i recovery in 14% CO_2 (see below). Removal of external Na^+ under control conditions brought pH_i down 0.18 ± 0.02 pH units ($n = 7$) (Fig. 2A). In the absence of external Cl^-, an acidification of 0.16 ± 0.03 pH units ($n = 10$) was observed (Fig. 2B). These results provide experimental evidence for the operation of a Na^+-dependent H^+ efflux driven by a Na^+-H^+ exchanger and a HCO_3^--Cl^- exchanger under basal conditions.

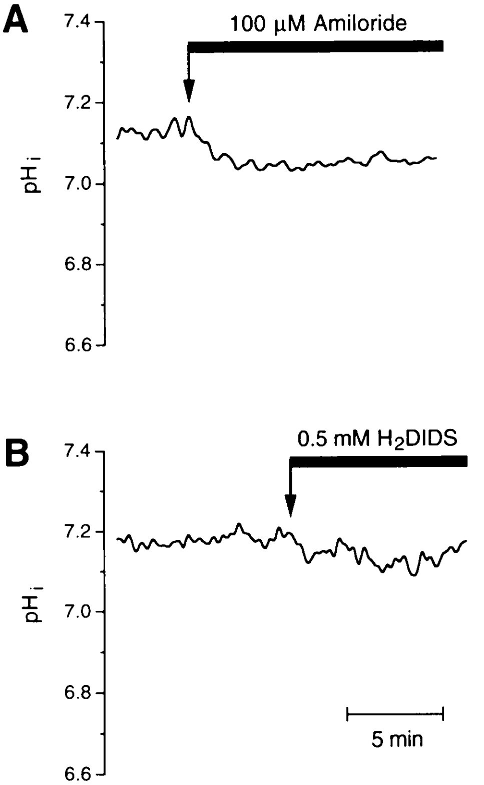

Figure 1. Effect of 100 μ*M* amiloride (A) and 0.5 m*M* H_2DIDS (B) on pH_i in piglet cerebral microvascular endothelial cells. pH_e was kept constant at 7.4 with a PCO_2 of 35 mm Hg. Identical experiments were performed on five (A) and 10 (B) independent cultures in duplicate.

Effect of amiloride, H_2DIDS, and external Cl^- on pH_i recovery from an acid load evoked by Na propionate. As described in our previous publication (7), pH_i in piglet cerebral microvascular endothelial cells returned fully to basal values within 10 min after administration of the weak acid Na propionate, as shown with dashed lines in Figure 3. Recovery of pH_i after intracellular acidification by Na propionate was then observed in the presence of amiloride. Figure 3A shows pH_i in endothelial cells acidified with 80 m*M* Na propionate fell to 6.82 ± 0.02 ($n = 5$) and recovered to a final pH_i of 7.14 ± 0.02 in the presence of 25 μ*M* amiloride. When the amiloride concentration was increased to 50 μ*M*, the cells were acidified to 6.78 ± 0.04 ($n = 5$) and recovered to a final pH_i of 6.90 ± 0.03 (Fig. 3B). With 100 μ*M* amiloride administration, the cell pH_i decreased to 6.70 ± 0.03 ($n = 5$) with a final pH_i of 6.82 ± 0.02 (Fig. 3C).

Recovery of pH_i after intracellular acidification by Na propionate was tested further by using H_2DIDS, a blocker of the anion exchanger. When the cell pH_i was decreased to 6.80 ± 0.04 ($n = 5$) by Na propionate,

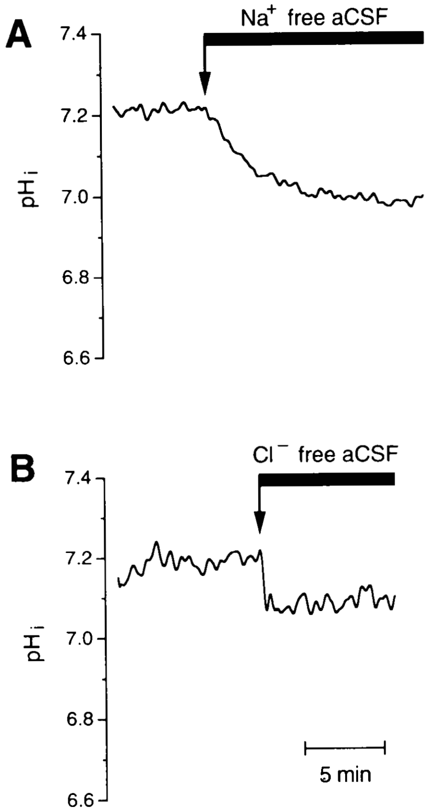

Figure 2. Effects of Na$^+$-free medium (A) and Cl$^-$-free medium (B) on pH$_i$ in piglet cerebral microvascular endothelial cells. pH$_e$ was kept constant at 7.4 with a PCO$_2$ of 35 mm Hg. Identical experiments were performed on five (A) and seven (B) independent cultures in duplicate.

H_2DIDS (0.5 m*M*) only partially inhibited the pH$_i$ recovery, as shown in Figure 4. Similarly, removal of Cl$^-$ partially blunted the pH$_i$ recovery after Na propionate (Fig. 4B). The combination of 0.5 m*M* H_2DIDS and 25 μ*M* amiloride further inhibited pH$_i$ recovery after Na propionate (Fig. 5) ($n = 5$). These results suggest that pH$_i$ recovery from a weak acid load depends more on the operation of the Na$^+$-H$^+$ exchanger than on that of the HCO$_3^-$-Cl$^-$ exchanger.

Effect of amiloride, H_2DIDS, and external Na$^+$ on pH$_i$ recovery from an acid load evoked by hypercapnia. When endothelial cells were acidified with 14% CO$_2$, pH$_e$ 7.4 in the presence of 100 μ*M* of amiloride, pH$_i$ fell to 6.85 ± 0.02 ($n = 7$) and stayed low with the continual perfusion of amiloride (Fig. 6A). In the absence of amiloride, the pH$_i$ returned to normal levels, as described in our previous publication (7) and as shown as dashed lines in Figure 6. The effects of H_2DIDS were virtually identical to those of amiloride (i.e., no recovery of pH$_i$ [6.88 ± 0.02 ($n = 7$)] during perfusion with 14% CO$_2$, pH$_e$ 7.4) (Fig. 6B). Removal of external Na$^+$ also completely abolished pH$_i$ recovery after an acid load evoked by 14% CO$_2$, pH$_e$ 7.4 ($n = 7$), as shown in Figure 7. These data show that both the amiloride-sensitive Na$^+$-H$^+$ exchanger and the Na$^+$-dependent H_2DIDS-sensitive HCO$_3$-Cl$^-$ exchanger are involved in restoration of pH$_i$ during hypercapnia.

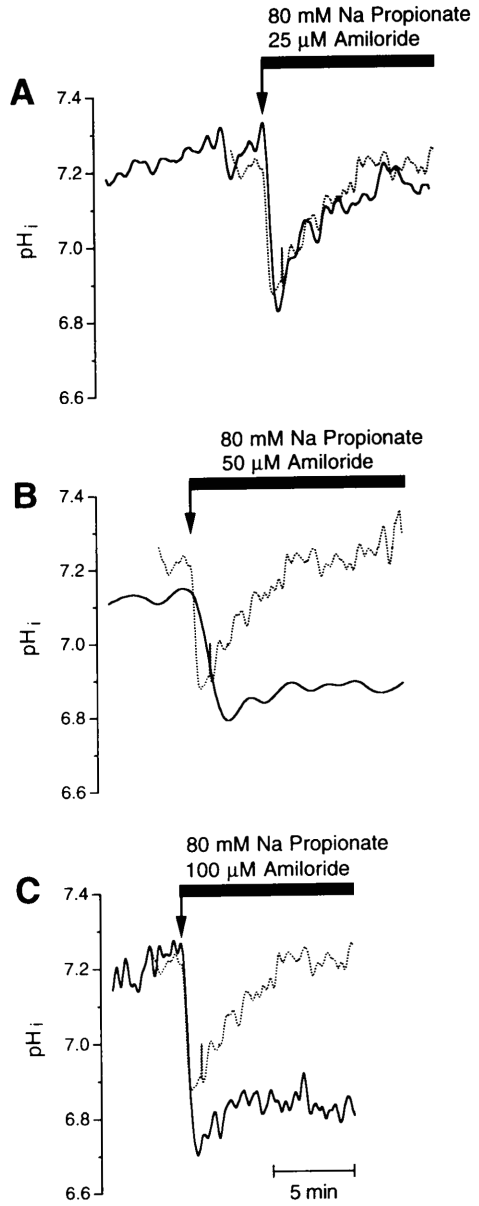

Figure 3. Effects of 25 μ*M* amiloride (A), 50 μ*M* amiloride (B), and 100 μ*M* amiloride (C) on pH$_i$ recovery after 80 m*M* Na propionate in piglet cerebral microvascular endothelial cells. pH$_e$ was kept constant at 7.4 with a PCO$_2$ of 35 mm Hg. Dashed lines represent pH$_i$ recovery from acid loading in the absence of amiloride (other cells). Solid traces represent pH$_i$ recovery from acid loading in the presence of amiloride. Identical experiments were performed on five independent cultures in duplicate.

Discussion

The endothelium is not just a simple lining of the intimal surface of blood vessels. Endothelial cells can sense mechanical, chemical, and humoral stimuli, process these signals, and respond by the synthesis and release of a variety of vasoactive factors (1). These substances can play an anticoagulating role in physiologic homeostasis and affect the tone of the adjacent

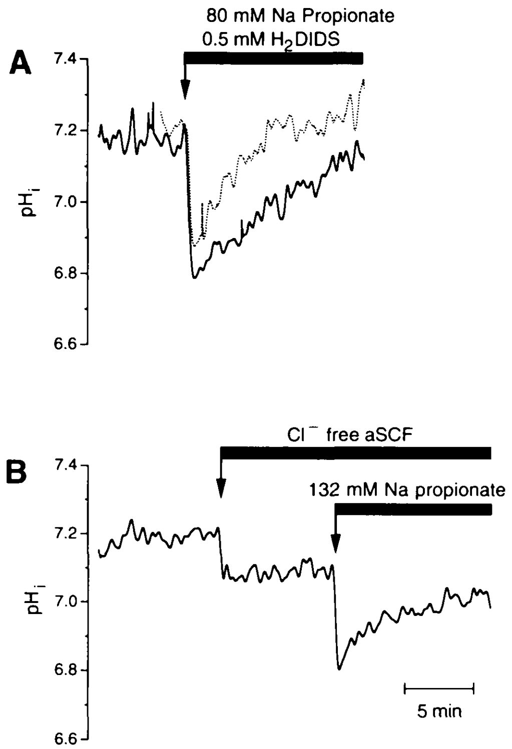

Figure 4. Effects of 0.5 m*M* H_2DIDS (A) or Cl^--free medium (B) on pH_i recovery after 80 m*M* Na propionate in piglet cerebral microvascular endothelial cells. pH_e was kept constant at 7.4 with a PCO_2 of 35 mm Hg. Dashed lines represent pH_i recovery from acid loading in the absence of H_2DIDS (other cells). Solid traces represent pH_i recovery from acid loading in the presence of H_2DIDS. Identical experiments were performed on five independent cultures in duplicate.

smooth muscle. Cerebral microvasculature endothelial cells form the BBB, which is essential to the stability of the cerebral microenvironments.

The present study suggests that the Na^+-H^+ exchanger and the HCO_3^--Cl^- exchanger are major contributors to pH_i regulation in piglet cerebral microvascular endothelial cells. pH_i recovery after a weak acid load is dependent primarily on Na^+-H^+ exchange activity and to a lesser extent on HCO_3^--Cl^- exchange activity, while pH_i recovery from high CO_2 is

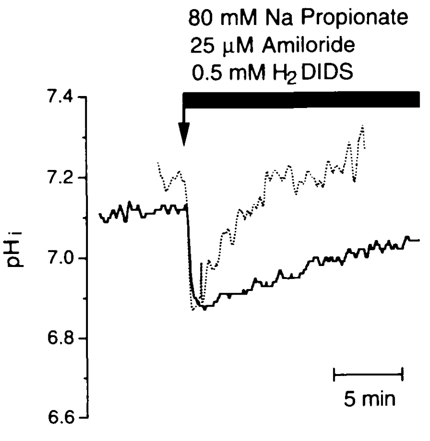

Figure 5. Effects of 0.5 m*M* H_2DIDS and 25 μ*M* amiloride on pH_i recovery after 80 m*M* Na propionate in piglet cerebral microvascular endothelial cells. pH_e was kept constant at 7.4 with a PCO_2 of 35 mm Hg. The dashed line represents pH_i recovery from acid loading in the absence of amiloride and H_2DIDS (other cells). The solid trace represents pH_i recovery from acid loading in the presence of amiloride and H_2DIDS. Identical experiments were performed on five independent cultures in duplicate.

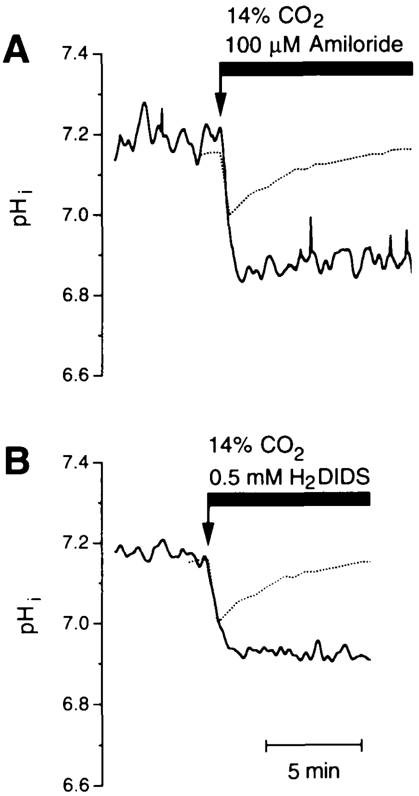

Figure 6. Effects of 100 μ*M* amiloride (A) and 0.5 m*M* H_2DIDS (B) on pH_i recovery after 14% CO_2 in piglet cerebral microvascular endothelial cells. pH_e was kept constant at 7.4 with a PCO_2 of 35 mm Hg. Dashed lines represent pH_i recovery from acid loading in the absence of amiloride or H_2DIDS (other cells). Solid traces represent pH_i recovery from acid loading in the presence of amiloride or H_2DIDS. Identical experiments were performed on seven independent cultures in duplicate.

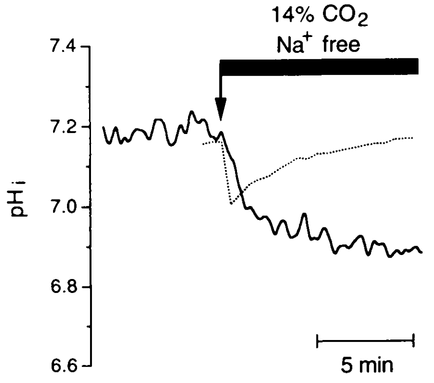

Figure 7. Effects of Na^+-free medium on pH_i recovery after 14% CO_2 in piglet cerebral microvascular endothelial cells. pH_e was kept constant at 7.4 with a PCO_2 of 35 mm Hg. The dashed line represents pH_i recovery from acid loading in the presence of Na^+ (from Ref. 7). The solid trace represents pH_i recovery from acid loading in the absence of Na^+. Identical experiments were performed on seven independent cultures in duplicate.

dependent upon activities of both the Na^+-H^+ and HCO_3^--Cl^- exchangers.

Decreases in pH_i may impair cellular functions such as enzyme activities and affect the cellular structure (6). During metabolic and respiratory acidosis, endothelial cells encounter the pH change. Acidosis caused by metabolic and respiratory disease or subsequent to eventful birth is a constant threat to the newborn. Therefore, it is valuable to know how the cerebral endothelial cells extrude H^+ when challenged by an acid load. In the present study of microvascular endothelial cells from newborn pig brain in primary culture, we found that endothelial cells extrude H^+ by an amiloride-sensitive Na^+-H^+ exchanger and a Na^+-dependent, H_2DIDS-sensitive HCO_3^--Cl^- exchanger even during basal conditions. After an acid load from a weak organic acid, the cells employed predominantly the Na^+-H^+ exchanger to recover pH_i. After an acid load from hypercapnia, both the Na^+-H^+ exchanger and the HCO_3^--Cl^- exchanger are necessary. Thus, it appears that these exchangers in cerebral microvascular endothelial cells may have important physiological roles in protecting cells from intracellular acidification during metabolic and respiratory acidosis.

Maintenance of pH_i in endothelial cells involves a Na^+-H^+ exchanger and an anion exchanger, although to a lesser extent. The Na^+-H^+ exchanger is amiloride-inhibitable (Fig. 1A), while the Na^+-dependent anion exchanger is H_2DIDS-sensitive (Fig. 1B). Both of these transporters are Na^+ dependent, since Na^+-free media decreased pH_i (Fig. 2A) to the extent caused by amiloride and H_2DIDS together. That the anion exchanger is a HCO_3^--Cl^- exchanger was suggested by the Cl^-free acidification (Fig. 2B) and inhibitory effect by the stilbene derivative H_2DIDS (Fig. 2). The acidification produced by Cl^--free medium was surprising since alkalization was expected. We believe these data suggest extracellular Cl^- is necessary for exchanger function, thereby preventing HCO_3^- uptake by this mechanism in the absence of extracellular Cl^-. This interpretation is speculative. The involvement of a Na^+-HCO_3^- cotransporter cannot be ruled out by the present study. These observations were comparable to what were found in Fu5, a rat hepatoma cell (11), in rabbit esophageal cells (12, 13), in rat zona glomerulosa cells (14), and in human thyroid epithelial cells (15).

Both the Na^+-H^+ exchanger and the HCO_3^--Cl^- exchanger are essential for pH_i restoration after a weak acid load. When cells were acidified utilizing Na propionate, recovery of pH_i occurred within 10 min, as shown in our previous study (7), but was inhibited when either amiloride or H_2DIDS was present or external Cl^- was depleted (Fig. 3 and 4). However, amiloride was a stronger blocker of pH_i recovery than H_2DIDS, and the combination of both showed an additive inhibitory effect (Fig. 5). These data suggest that Na^+-H^+ exchange activity contributed more to pH_i restoration after a weak acid load than HCO_3^--Cl^- exchange activity did. Na^+-H^+ exchange and HCO_3^--Cl^- exchange systems have been detected in many cell types and are responsible for pH_i recovery (9, 13, 15, 16). In some cell types, such as rat sublingual mucous acini (17) and human fibroblasts (18), only Na^+-H^+ exchangers are needed for recovery of pH_i after an acid load and are more active at acidic pH (19). In rat pyramidal neurons, the major acid extrusion mechanism is the Na^+-dependent HCO_3^--Cl^- exchanger (20). In rabbit pulmonary macrophages, H^+ extrusion is accomplished predominantly by a H^+-ATPase (5, 21, 22). Certainly proton transporters other than Na^+-H^+ exchangers and HCO_3^--Cl^- exchangers may also be present in endothelial cells. It appears that pH_i recovery after an acid load is cell type and possibly species dependent.

pH_i recovery after hypercapnia also depended on both the Na^+-H^+ exchanger and the HCO_3^--Cl^- exchanger. When intracellular acidification was induced by high CO_2, restoration of pH_i was entirely blocked by either amiloride (Fig. 6A), or H_2DIDS (Fig. 6B). The endothelial cells needed the presence of Na^+ to recover normal pH_i after hypercapnia (Fig. 7). Our results indicate that both the Na^+-H^+ exchanger and the Na^+-dependent HCO_3^--Cl^- exchanger are active and can interactively regulate pH_i to the resting level during hypercapnia. Hypercapnia in this study as an acid challenge would be similar to compensated respiratory acidosis. Nakanishi *et al.* (23) have shown in rabbits that the decrease in myocardial pH_i during respiratory acidosis in the newborn was less than that in the adult, basically because the HCO_3^--Cl^- exchange activity is more active in the premature myocardium. Whether age-specific differences exist in pigs regarding maintenance of microvascular endothelial cell pH is not known as these data are not available from cells of older pigs. Our studies also suggest that the HCO_3^--Cl^- exchanger is more active than cerebral endothelial cells from newborn pigs are exposed to high CO_2 than when they are exposed to a weak acid.

In summary, we have demonstrated that acid-extruding mechanisms in piglet cerebral microvascular endothelial cells include an amiloride-sensitive Na^+-H^+ exchanger and a Na^+-dependent H_2DIDS-sensitive HCO_3^--Cl^- exchanger. The contributions of these two exchangers to pH_i are dependent upon the types of acid challenges.

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