Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 522–526, January 1986
Physiological Sciences

Optical measurements of intracellular pH in single LLC-PK_1 cells: Demonstration of Cl–HCO_3 exchange

(Na–H exchange/pH-sensitive dyes)

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Communicated by Edward A. Adelberg, August 28, 1985

ABSTRACT **An optical method was used to continuously monitor intracellular pH (pH_i) in single cultured LLC-PK_1 cells. Rapidly growing or quiescent cells, attached to coverslips, were loaded with the pH-sensitive dye 4',5'-dimethyl-5(and -6)-carboxyfluorescein by exposing them to the dye's permeant precursor. pH_i was calculated from the intracellular absorbance spectrum of the dye in a single cell. For cells incubated in HCO_3^--free Ringer's solution, pH_i recovered exponentially from acid loads applied by NH_4^+ prepulsing. Because the recovery was Na^+-dependent and amiloride-sensitive, it was probably caused by Na–H exchange at the plasma membrane. In HCO_3^- Ringer's solution, external Cl^- removal caused pH_i to reversibly increase by ≈0.3. This pH_i increase was substantially reduced by 50 μM 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) or by conducting the Cl^- removal in the nominal absence of HCO_3^-. Reducing $[HCO_3^-]_o$ from 25 to 5 mM at constant pCO_2 (lowering pH_o from 7.4 to 6.7) caused pH_i to reversibly fall by ≈0.2. This pH_i change was greatly diminished by DIDS, by removal of extracellular Cl^-, or by performing the same pH_o shift in the nominal absence of HCO_3^-. The pH_i changes induced by altering $[Cl^-]_o$ or pH_o were not inhibited by Na^+ removal. Our data indicate that LLC-PK_1 cells possess a Na-independent Cl–HCO_3 exchanger and that this transporter may be as important as the Na–H exchanger in determining pH_i.**

The appropriate regulation of intracellular pH (pH_i) is crucial because of the large number of pH-sensitive processes (refs. 1–3). Furthermore, controlled pH_i changes have been implicated in the action of insulin (4), the activation of eggs (5) and sperm (6), and the stimulation of cells by growth factors (7–10). A full appreciation of the role of pH_i changes in affecting cell function requires a detailed knowledge of the mechanisms by which pH_i is regulated, which, in turn, can only be assessed by measuring rapid pH_i changes. pH-sensitive microelectrodes (11, 12) are useful for examining pH_i regulation in relatively large cells. With the advent of reliable pH-sensitive dyes, studies of pH_i regulation have been extended to cells in culture, though only on large populations of cells. Recently, a technique has been developed in our laboratory (13, 14) that permits the accurate measurement of rapid pH_i transients from a small group of cells in a single renal tubule. The intracellular absorbance spectrum of a pH-sensitive dye is measured by placing the cells in a narrow beam of light, and pH_i is calculated from the shape of this spectrum. We decided to employ this approach for studying pH_i regulation in single cultured cells of the LLC-PK_1 renal epithelial cell line (15), which grows normally and can be induced to differentiate under specified conditions.

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pH_i regulation was first studied in large invertebrate cells that control pH_i by exchanging external Na^+ and HCO_3^- for internal Cl^- and possibly H^+ (see refs. 1 and 2). This Na/HCO_3–Cl/H exchange activity is blocked by 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) and related stilbenes and is not significantly affected by amiloride. On the other hand, the predominant pH_i-regulating system of many vertebrate cells exchanges external Na^+ for internal H^+. This Na–H exchanger is insensitive to stilbenes but is inhibited by amiloride. The possible role of HCO_3^- transport in the pH_i homeostasis of vertebrate cells has received relatively little attention. Recent studies on epidermoid carcinoma (10) and fibroblast cell lines (16) indicate that pH_i regulation may occur by Na/HCO_3–Cl/H exchange and Na–H exchange. The effect on pH_i of a Na-independent Cl–HCO_3 exchanger has been demonstrated in sheep cardiac Purkinje fibers (17) and in the amphibian gallbladder (18). However, a possible pH_i-regulatory role for a simple Cl–HCO_3 exchanger has yet to be investigated either in cultured cells or in mammalian epithelial cells.

Work by others suggests the presence of a Na–H exchanger in LLC-PK_1 cells. Amiloride inhibits ^{22}Na uptake by confluent PK_1 cells (19) and by rapidly growing LLC-PK_1 cells that have been acid loaded (20). In this paper we demonstrate directly that LLC-PK_1 cells do indeed regulate pH_i by means of a Na–H exchanger. In addition, we show that these cells also possess a Cl–HCO_3 exchanger that functions independently of Na^+. Moreover, under the experimental conditions employed in this study, maximal rates of Cl–HCO_3 exchange were several times higher than those of Na–H exchange. This is consistent with the hypothesis that, like the Na–H exchanger, the Cl–HCO_3 exchanger plays a major role in determining pH_i.

MATERIALS AND METHODS

Solutions. Standard HCO_3^--free Ringer's solution had the following composition (in mM): 128 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 32.2 Hepes (titrated with NaOH to pH 7.4), 5.5 glucose, 5 alanine. It was equilibrated with air and had an osmolality of ≈300 mosmol/kg. In Na-free Ringer's solution, Na^+ was replaced mol-for-mol with *N*-methyl-D-glucamine$^+$. In 20 mM NH_4^+ Ringer's solution, NH_4^+ replaced Na^+ mol-for-mol. For experiments in which the Ringer's solution was buffered to pH 7.4 or 6.7 with 2 mM Pipes, additional NaCl was added to maintain the osmolality. In standard HCO_3^- Ringer's solution (pH 7.4), 25 mM HCO_3^- replaced the

Abbreviations: pH_i, intracellular pH; pH_o, extracellular pH; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonate; Me_2CF, 4',5'-dimethyl-5(and -6)-carboxyfluorescein.

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NaHepes buffer, and the solutions were equilibrated with 5% CO_2/95% air. In the pH 6.7 HCO_3^- Ringer's solution, [HCO_3^-] was lowered to 5 mM, with Cl^- replacing HCO_3^-. Temperature was always 37°C, and external pH (pH_o) was 7.40 unless otherwise indicated. Solutions were delivered by gravity through CO_2-impermeable tubing.

Cells and Culture Conditions. Clone 4 of LLC-PK_1 cells (obtained from J. S. Cook, Oak Ridge National Laboratory, Oak Ridge, TN) was maintained at subconfluent density in αMEM medium (21) supplemented with 10% fetal calf serum and incubated with 5% CO_2 at 37°C. Cells (10^4) contained in 2 ml of this medium were seeded in a 35-mm tissue culture dish containing a 1 cm × 1 cm sterile glass coverslip. "Rapidly growing" cells, attached to coverslips, were used 2 days later. "Quiescent cells" were obtained by aspirating the culture medium and replacing it with serum-free medium every 2 days. Quiescent cells were obtained 8 days after seeding, at which time >95% of the cells were quiescent, as determined by [^{3}H]thymidine incorporation and by progression of cells into M phase (22).

Measurement of pH_i. Ringer's solution (37°C) continuously flowed through a chamber mounted on the stage of an inverted microscope. The coverslip holding the cells formed the chamber's bottom and was attached to the underside of the chamber with vacuum grease and screws. Cells were loaded with 4',5'-dimethyl-5(and -6)-carboxyfluorescein (Me_2CF) by exposing them to the dye's permeant diacetate derivative (Molecular Probes, Junction City, OR), which is cleaved by esterases to yield the relatively impermeant Me_2CF. A single cell containing Me_2CF was illuminated with a spot of white light 10 μm in diameter, and the transmitted light was projected on a diffraction grating. As described previously (13), the resulting spectrum, between wavelengths of 400 and 800 nm, was focused on a 1024-element photodiode array (E.G. & G., Salem, MA/Princeton Applied Research, Princeton, NJ) that was interfaced to a DEC 11/23-based computer. Absorbance spectra of intracellular Me_2CF (corrected for the absorbance of the cell) were obtained about once every 3 sec. pH_i was calculated from the ratio of dye absorbance at 510 nm (the wavelength of peak absorbance) to that at 470 nm (the *in vitro* isosbestic wavelength). An intracellular calibration of this absorbance ratio was obtained by recording absorbance spectra of intracellular Me_2CF as pH_i was varied by altering pH_o in a Ringer's solution containing 10 μM nigericin and a high level of K^+, an approach used by Thomas *et al.* (23). In the absence of data on $[K^+]_i$ for LLC-PK_1 cells, we chose a value of 105 mM for $[K^+]_o$, the same as we used previously for the rabbit renal cortical collecting tubule (14). The dependence of the absorbance ratio on pH_i was described by a pH titration curve having a pK of 7.28.

RESULTS

The following experiments generally were performed on both rapidly growing and quiescent cells. The results were qualitatively the same for both growth states.

Na–H Exchange. To test for the presence of a Na–H exchanger, we monitored the recovery of pH_i from an acute acid load in nominally HCO_3^--free solutions. The initial pH_i values of rapidly growing and quiescent cells in standard HCO_3^--free Ringer's solution were not significantly different, averaging 7.27 ± 0.05 (mean ± SEM, n = 14 cells). In the experiment of Fig. 1, the cell is acid loaded by a brief exposure to Ringer's solution containing 20 mM NH_4^+ (12, 24). Application of NH_4^+ causes a rapid pH_i increase (segment ab), due to the influx and protonation of NH_3, followed by a slower pH_i decrease (segment bc), due to the influx and dissociation of the less-permeant NH_4^+. Removal of external NH_4^+ causes a large pH_i decrease (segment cd) as intracellular

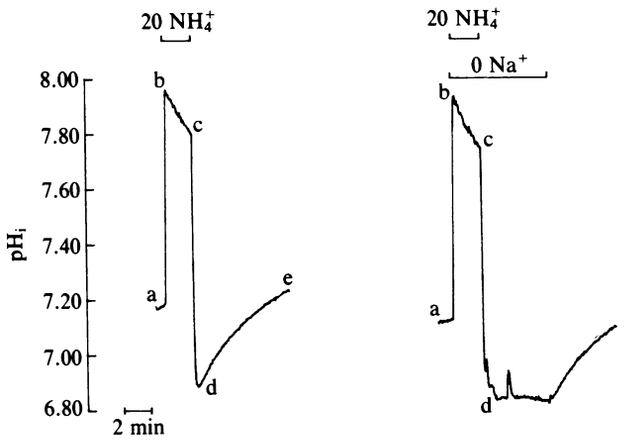

FIG. 1. Response of a single, quiescent LLC-PK_1 (Cl4) cell to intracellular acid loading. Similar results were obtained with rapidly growing cells.

NH_4^+ dissociates into NH_3 (which exits the cell) and H^+ (which is trapped inside). In the presence of extracellular Na^+, pH_i recovers from the acid load (segment de), due to a pH_i-regulating mechanism. However, when the acid load is applied in the absence of external Na^+ (Fig. 1 *Right*) pH_i fails to recover until Na^+ is reapplied. For 6 cells in which we measured pH_i recovery rates in 0 and 140 mM Na^+ at similar pH_i values, Na^+ removal inhibited pH_i recovery by 88.3% ± 4.3%. The small Na^+-independent component could result from leakage of the Hepes buffer, metabolism, or some other transport process. The pH_i recovery rate in 29 mM Na^+, compensating for the small Na^+-independent component, was 26% ± 3% (n = 3), as large as that in 145 mM Na^+.

The recovery of pH_i from an NH_4^+-induced acid load was reversibly inhibited by amiloride. In six cells acid loaded in Na^+-free Ringer's solution (as in Fig. 1 *Right*) containing 1 mM amiloride, addition of 29 mM Na^+ did not noticeably increase the rate of pH_i recovery. Our observations are consistent with the earlier finding that ^{22}Na uptake by rapidly growing PK_1 cells is stimulated by intracellular acid loading and is inhibited by amiloride analogs (20) and indicate that rapidly growing and quiescent LLC-PK_1 (Cl4) cells can regulate pH_i by means of a Na–H exchanger.

Intracellular Buffering Power. The data of Fig. 1 can also be used to compute the cell's intrinsic buffering power (β_I)—that is, the buffering power that prevails in the absence of exogenous buffers (e.g., CO_2). By definition, buffering power is the ratio of the applied intracellular acid load (given in mM) to the resultant pH_i decrease. The magnitude of the acid load in this experiment is the amount of H^+ released as NH_4^+ dissociates into NH_3 and exits the cell after the removal of external NH_4^+ (point c) and is easily calculated (see ref. 25). The resultant pH_i decrease is the pH_i change between points c and d. In 12 experiments in standard HCO_3^--free Ringer's solution, β_I was 6.2 ± 0.5 mM. As far as we are aware, this value of β_I is the lowest reported for any cell (see ref. 1). The results were the same in Na^+-free Ringer's solution, suggesting that the reduction of the very rapid segment cd pH_i decline by Na–H exchange is normally minimal in these cells. In Ringer's solution containing 10 mM acetate, the apparent β_I was appreciably higher (i.e., 14.4 ± 0.6 mM, n = 2), possibly due to the extra buffering provided by an open-system acetate/acetic acid buffer pair (see ref. 1).

Cl–HCO_3 Exchange: pH_i Response to Altered $[Cl^-]_o$. Experiments designed to examine HCO_3^- transport were always begun in Ringer's solution buffered to pH 7.4 with 25 mM HCO_3^- and 5% CO_2. The initial pH_i values for rapidly growing and quiescent cells in standard HCO_3^--containing Ringer's solution were not significantly different, averaging 7.14 ±

0.03 (n = 26), significantly lower ($P < 0.01$) than the value of 7.27 for cells in HCO_3^--free Ringer's solution (see above). Such a difference in steady-state pH_i values implies that cells in HCO_3^- Ringer's solution are subject to a greater, chronic intracellular acid load, as would be expected if a transporter (e.g., Cl–HCO_3 exchanger) mediated a continuing net efflux of HCO_3^-.

At point a in the experiment of Fig. 2, all external Cl^- is replaced with glucuronate, causing pH_i to increase by 0.29 over a 4.8-min period (segment ab). Return of Cl^- has the opposite effect (segment bc). These pH_i changes are consistent with a plasma-membrane Cl–HCO_3 exchanger, which should respond to external Cl^- removal by driving HCO_3^- into the cell. As shown in the latter half of Fig. 2, the stilbene derivative DIDS (50 μM), which inhibits HCO_3^- transport in other systems, greatly reduces the magnitude and the rate of intracellular alkalinization induced by Cl^- removal (segment cd). Deletion of the DIDS at a time (point d) when the drug's effect is still partially reversible allows pH_i to increase further (segment de). In 19 experiments, the pH_i increase produced by Cl^- removal averaged 0.32 ± 0.03, significantly greater than in the presence of DIDS, 0.16 ± 0.07 (n = 3, see Table 1). In paired experiments, DIDS reduced the initial rate of pH_i decrease caused by the readdition of Cl^- by 74% ± 8% (n = 3).

In a second set of experiments (not shown), the effect of removing external Cl^- was compared in HCO_3^- vs. nominally HCO_3^--free media. HCO_3^- removal reduced the pH_i change by ≈62% (see Table 1). However, the effect on the flux of acid/base equivalents must have been even greater, inasmuch as intracellular buffering power is markedly reduced by HCO_3^- removal.** In three paired experiments, HCO_3^- removal reduced the flux of acid/base equivalents by 91% ± 2%. Thus, the transport system appears to be dependent on HCO_3^- per se. Finally, we examined the Na^+ dependence of the response to Cl^- removal. In three cells, the pH_i increase induced by Cl^- removal in the absence of Na^+ was 0.44 ± 0.04, somewhat higher than the value of 0.32 obtained in the presence of Na^+. Thus, the HCO_3^- transporter responsible for these pH_i changes is capable of functioning in the absence of Na^+.

Cl–HCO_3 Exchange: pH_i Response to Altered $[HCO_3^-]$. If the pH_i changes induced by altering $[Cl^-]_o$ are indeed due to Cl–HCO_3 exchange, pH_i should be sensitive to changes in $[HCO_3^-]_o$. Fig. 3 illustrates an experiment in which $[HCO_3^-]_o$ was reduced from 25 to 5 mM at a constant pCO_2 of 5% (i.e., reducing pH_o from 7.4 to 6.7). This causes a slow fall in pH_i of ≈0.14 (segment ab) that is reversed upon returning the cell to the original pH 7.4 solution (segment bc). In eight similar experiments, the mean pH_i change over a period of ≈4 min was 0.19 ± 0.02 (see Table 1). To determine whether these pH_i changes were due to HCO_3^- fluxes, the same pH_o changes were made after replacing CO_2/HCO_3^- with Pipes buffer. The effects on pH_i of Pipes fluxes were minimized by reducing $[Pipes]_o$ to 2 mM. Changing from pH 7.4 CO_2/HCO_3^- to pH 7.4 Pipes Ringer's solution causes a rapid rise in pH_i (segment cd) due to the exit of CO_2 from the cell. In eight experiments, CO_2/HCO_3^- removal caused a sustained pH_i increase of 0.19 ± 0.02. When pH_o is now reduced from 7.4 to 6.7, pH_i falls by a smaller amount (0.03 vs. 0.14) than it had in the presence of HCO_3^- (segment de), and the initial rate of pH_i change corresponds to a flux of acid/base equivalents only 3% as great as under control conditions.** If the pH_i changes throughout segment abc had been due to flux of OH^-, for example, then one might have expected the segment def pH_i changes to be larger, inasmuch as the initial $[OH^-]_i$ was higher. After the cell is returned to CO_2/HCO_3^- Ringer's solution (segment fg), pH_o changes once again produce larger and more rapid pH_i shifts. Thus, HCO_3^- fluxes are probaby the primary cause of the pH_i changes induced by simultaneously altering pH_o and $[HCO_3^-]_o$.

Another prediction of the Cl–HCO_3-exchange hypothesis is that pH_i changes induced by altering $[HCO_3^-]_o$ should occur only in the presence of Cl^-. In the first part of Fig. 4 (segment ab), simultaneous reduction of pH_o and $[HCO_3^-]_o$ elicits a reversible pH_i fall, as in Fig. 3. Subsequent removal of all Cl^- from the external solution (segment cd) causes pH_i to increase by 0.38, presumably due to Cl–HCO_3 exchange (see Fig. 2). Because $[HCO_3^-]_i$ is now substantially elevated, one might expect reduction of $[HCO_3^-]_o$ to elicit a HCO_3^- efflux that is greater than normal. Instead, when pH_o and $[HCO_3^-]_o$ are now reduced simultaneously in the nominal absence of Cl^-, the pH_i decline (segment def) is much slower and smaller than in the previous (segment abc) or succeeding (segment ghi) controls (see Table 1). The possibility that the high pH_i prevailing throughout segment def could have directly inhibited Cl–HCO_3 exchange is highly unlikely, given the observation (26) that the HCO_3^- efflux mediated by a Cl–HCO_3 exchanger is enhanced by acute alkali loads in Purkinje fibers. The small pH_i changes remaining in the absence of Cl^- (segment def) could be due to the presence of residual Cl^- or to the movement of acid/base equivalents via a parallel transporter. The pH_i change elicited by altering $[HCO_3^-]_o$ was also inhibited by DIDS (Table 1). In two paired experiments, 50 μM DIDS reduced the pH_i change from 0.17 ± 0.02 to 0.03 ± 0.01 ($P < 0.01$).

If the pH_i changes of Figs. 3 and 4 were due to a Na/HCO_3–Cl/H exchanger, then these changes should be blocked by Na^+ removal. However, we found that Na^+ removal did not reduce the pH_i changes elicited by lowering $[HCO_3^-]_o$ (Table 1).

**The total buffering power (β_T) is β_I plus β due to CO_2 (β_{CO_2}). For a cell in HCO_3^--free Ringer's solution, where $\beta_{CO_2} = 0$, $\beta_T = \beta_I \simeq$ 6 mM. For the same cell incubated in 5% CO_2 and at a pH_i of 7.1, $\beta_{CO_2} \simeq 29$ mM, assuming a constant pCO_2. Thus, $\beta_T = 6 + 29 =$ 35 mM, nearly six times higher than in the absence of HCO_3^-.

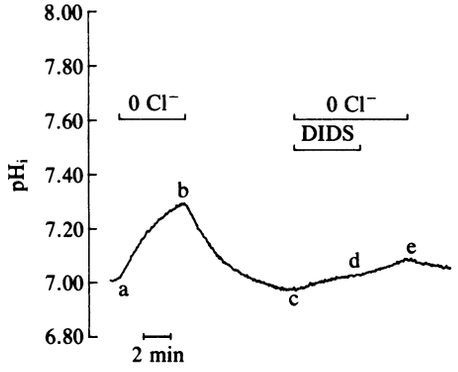

FIG. 2. Effect of Cl^- removal on pH_i in a single, rapidly growing cell exposed to HCO_3^--containing Ringer's solution. Similar results were obtained with quiescent LLC-PK_1 (Cl4) cells.

DISCUSSION

Earlier work on isotopic Na^+ fluxes (19, 20) strongly suggested that LLC-PK_1 cells possess a Na–H exchanger. The data of Fig. 1 confirm this hypothesis and show that this exchanger functions as a pH_i regulator as well. The pH_i recovery from the acute, NH_4^+-induced acid load was most rapid at low pH_i values and slowed as pH_i approached its initial level. Part of this slowing may have been due to an increase in $[Na^+]_i$ that Na–H exchange probably causes. However, given a β_I of 6 mM and a pH_i recovery of 0.4, the $[Na^+]_i$ increase would only have been 2.4 mM, even assuming no Na/K pump activity. If it is assumed that β_I is constant

Table 1. pH_i changes caused by altering $[Cl^-]_o$ or $[HCO_3^-]_o$

Maneuver	Control	Na^+-free	HCO_3^--free	Cl^--free	DIDS
$[Cl^-]_o$: 135 to 0 mM	0.32 ± 0.03 (19)	0.44 ± 0.04 (3)	0.12 ± 0.03 (9)	—	0.16 ± 0.07 (3)
pH_o: 7.4 to 6.7	0.19 ± 0.02 (8)	0.23 ± 0.02 (5)	0.03 (1)	0.04 ± 0.02 (5)	0.06 ± 0.04 (3)

pH_i changes (mean ± SEM; number of cells in parentheses) were measured 4–5 min after solution change. Paired and unpaired data are presented, with data on quiescent and rapidly growing cells combined. Differences between control and Na^+-free conditions are not statistically significant [$P > 0.05$], whereas differences between control and HCO_3^--free, Cl^--free, and DIDS conditions are statistically significant [$P < 0.05$]. Control conditions: pH_o = 7.4, $[Na^+]_o$ = 153 mM, $[Cl^-]_o$ = 135 mM, $[HCO_3^-]_o$ = 25 mM. When DIDS was present, $[DIDS]_o$ = 50 μM.

over the relevant pH_i range and $[Na^+]_i$ changes are negligible, then the exponential pH_i recovery implies that the Na–H exchange rate is linearly but inversely related to pH_i. Such inverse-linear dependence of acid-extrusion rate on pH_i is observed consistently (see refs. 1 and 2) for Na–H and Na/HCO_3–Cl/H exchangers and is probably due to an intracellular H^+-binding modifier site on the exchangers (27). The Na–H exchanger's threshold, the pH_i at which the acid-extrusion rate apparently falls to zero (28), does not necessarily equal the steady-state pH_i following recovery from an acid load. Such an equality is achieved only if the acid loading rate is zero. Although a zero acid-loading rate is never exactly achieved, it is probably approached when cells are incubated in HCO_3^--free Ringer's solution, in which case acid loading by HCO_3^- efflux would be minimal. Haggerty *et al.* (20) found that amiloride-sensitive ^{22}Na fluxes into LLC-PK_1 cells incubated in HCO_3^--free media are substantial when cells are acid loaded by an NH_4^+ prepulse but undetectable under control conditions (i.e., $pH_i \approx 7.3$). This suggests that Na–H exchangers are near their apparent threshold when cells are in a steady state in HCO_3^--free Ringer's solution and, therefore, that the Na–H exchanger's pH_i threshold is ≈7.3.

Our demonstration of a Na–H exchanger in the rapidly growing cells is superficially at odds with the ^{22}Na flux data of Cantiello *et al.* (19), who deduced that Na–H exchange occurs in confluent but not exponentially growing LLC-PK_1 cells. Inasmuch as the present LLC-PK_1 line is heterogeneous (29), it is possible that our clone differs from theirs. However, even if the Na–H exchanger had been present in their exponentially growing cells, they may not have been able to detect it because they apparently did not stimulate the exchanger with an intracellular acid load.

The data obtained in HCO_3^- Ringer's solution are entirely consistent with a simple Cl–HCO_3 exchanger: (*i*) reduction of $[Cl^-]_o$ causes a pH_i rise that is HCO_3^- dependent and DIDS sensitive and (*ii*) reduction of pH_o causes a pH_i fall that is HCO_3^- and Cl^- dependent and DIDS sensitive. The Cl–HCO_3 exchanger must be distinguished from two other coupled HCO_3^- transport systems. (*i*) The Na/HCO_3–Cl/H exchanger is present in several invertebrate (30–33) and at least two vertebrate cells (10, 16). Although this transporter is DIDS sensitive and dependent on HCO_3^- and Cl^-, it has an absolute requirement for Na^+ and has a steep pH_i dependence that shuts it off at pH_i values above ≈7.3. In the present study, we found no Na^+ dependence and were able to drive pH_i above 7.4 by removing external Cl^- (Fig. 4). (*ii*) The electrogenic Na/HCO_3^- cotransporter is present in the basolateral membrane of the amphibian renal proximal tubule (34). This cotransporter, unlike the Cl–HCO_3 exchanger, has an absolute requirement for Na^+ and is independent of Cl^-. In the present study, however, we observed the opposite dependencies on Na^+ and Cl^-. Thus, HCO_3^- transport in LLC-PK_1 cells is mediated primarily by Cl–HCO_3 exchange. Neither of the two aforementioned Na-linked HCO_3^- transport systems is likely to make a substantial contribution to the regulation of pH_i, at least under the conditions of our experiments.

Our data thus support the existence of separate Na–H and Cl–HCO_3 exchangers in quiescent and rapidly growing LLC-PK_1 (Cl4) cells. Because in all experiments the cells were subconfluent, and therefore minimally differentiated or polarized (29, 35), it is impossible to address the issue of whether the two exchangers are present in the luminal and/or basolateral membranes. Inasmuch as Na–H and Cl–HCO_3 exchangers were found in cells that had yet to develop more differentiated transport activities, such as Na-dependent hexose transport (35), these pH_i-related transporters are probably part of a constellation of transporters necessary for cell viability and not merely for specialized functions of the differentiated cell. Na–H exchange, the activity of which is closely regulated by pH_i, is probably the key element for pH_i regulation in these cells. Cl–HCO_3 exchange, which is not known to be highly sensitive to pH_i changes, probably helps defend the cell against alkali loads. Our observation that removal of CO_2/HCO_3^- causes a sustained increase of pH_i indicates that the Cl–HCO_3 exchanger normally operates in the direction of net HCO_3^- efflux, thereby acid loading the

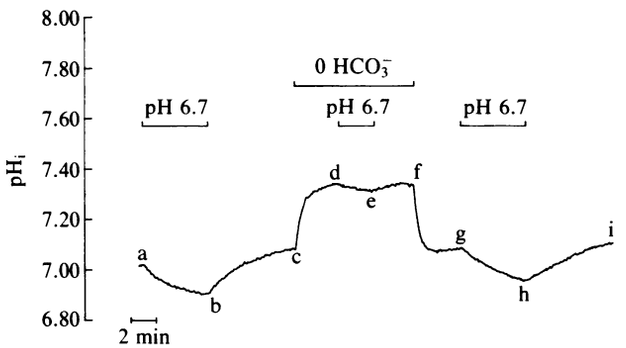

FIG. 3. Effect of lowering external pH (pH_o) in a single, rapidly growing cell: dependence on HCO_3^-.

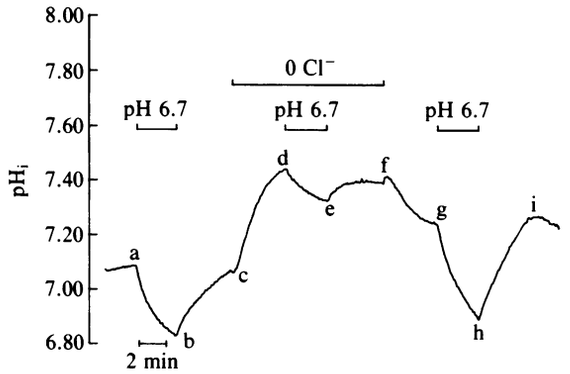

FIG. 4. Effect of lowering external pH in a single, rapidly growing cell: dependence on Cl^-. Similar results were obtained in quiescent LLC-PK_1 (Cl4) cells.

cell. In the normal steady state, acid loading by this and other mechanisms (e.g., metabolism) is balanced exactly by acid extrusion (i.e., Na–H exchange). During alkali loading, Na–H exchange would slow or even halt, and Cl–HCO_3 exchange would probably increase, thereby returning pH_i toward normal. During acid loading, Na–H exchange would increase, whereas Cl–HCO_3 exchange would probably slow or even reverse. The dynamic interaction between Na–H and Cl–HCO_3 exchangers in determining pH_i has yet to be investigated in any cell.

The relative importance of Na–H and Cl–HCO_3 exchange for establishing pH_i cannot be assessed directly from our data. Nevertheless, we can estimate their maximal contributions by comparing the rates of pH_i change mediated by the two exchangers. In Fig. 1, the average maximal (i.e., initial) rate of pH_i recovery from an acid load (segment de) was 0.079 pH unit/min. Given a β_I for this cell of 9.2 mM, this pH_i recovery rate corresponds to an acid-extrusion rate of 0.73 mM/min.

Comparable estimates of the Cl–HCO_3 exchange rate can be made from the data in Figs. 2–4. In the experiment of Fig. 2, return of extracellular Cl^- (point b) causes pH_i to fall at the rate of 0.102 pH unit/min. The equivalent HCO_3^- flux is the product of this rate of pH_i change and the total intracellular buffering power (β_T). β_T is the sum of β_I and β_{CO_2} (the buffering power of intracellular CO_2/HCO_3^-). Assuming a constant pCO_2, β_{CO_2} is $2.3 \cdot [HCO_3^-]_i$ (see ref. 1) and comes to 44.6 mM, so that β_T is 53.8 mM. The gross rate of acid loading is therefore (0.102 pH unit/min) × (53.8 mM) = 5.48 mM/min. From this must be subtracted 0.35 mM/min, the acid-loading rate in the presence of DIDS (at point e). The Cl–HCO_3 exchange rate, defined as the DIDS-sensitive acid-loading rate, is thus ≈5.13 mM/min. A similar analysis of Fig. 3 yields a HCO_3^--dependent acid loading rate of 1.54 mM/min. Inasmuch as the likely gradient for Na^+ (5:1 to 10:1 outside over inside; unpublished) exceeds that for H^+ (≈2:1), the Na–H exchanger must have been operating in the acid-extruding direction throughout the experiment. Thus, our value of 1.54 mM/min is probably an underestimate. Finally, an analysis of Fig. 4 yields a Cl-dependent component of acid loading of ≈6.4 mM/min.

In summary, when stimulated by an acid load of ≈0.3 pH unit, Na–H exchange proceeded at a rate of ≈0.7 mM/min. The Cl–HCO_3 exchange rate under three different conditions was 5.1, 1.5, and 6.4 mM/min. The Cl–HCO_3 exchanger is clearly capable of mediating acid/base fluxes greater than those mediated by the Na–H exchanger, with the latter operating under conditions of a fairly large acid load. This analysis must be interpreted with caution, inasmuch as the two exchangers were not assayed under identical conditions nor under the conditions that would prevail in the normal steady state. Furthermore, the Na–H exchange rate could possibly be increased by growth factors (8–10), by cell shrinkage (36, 37), or by further lowering pH_i. Nevertheless, our data suggest that Cl–HCO_3 exchange may be as important as Na–H exchange in determining pH_i in LLC-PK_1 cells. Although the possible contribution of Cl–HCO_3 exchange to pH_i regulation in cultured cells has heretofore not been appreciated, our data would suggest that Cl–HCO_3 should be seriously considered in future analyses of pH_i regulation in these and other cultured cells.

We thank Drs. E. A. Adelberg and A. W. Siebens for their comments on an earlier draft of this manuscript and E. Tyner and A. Yeatts for assisting in the data analysis. This research was supported by the Searle Scholars Program (W.F.B.), by National Institutes of Health Grants AM30344 (W.F.B.), AM01022 (W.F.B.), and CA34460 (E. Adelberg), and by National Science Foundation Grant PCM8306215 (E. Adelberg). J.R.C. was supported by a postdoctoral award from the Connecticut affiliate of the American Heart Association, and K.A. was supported by a Swebilius Cancer Research Award.

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