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Maintenance of Acidic Lateral Intercellular Spaces by Endogenous Fixed Buffers in MDCK Cell Epithelium

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Abstract. The lateral intercellular spaces (LIS) of MDCK cell epithelia grown on permeable supports are about 0.4 pH units acidic to the bathing solutions, presumably because of buffering by the fixed negative charges on the surface of the lateral cell membranes. To test the hypothesis that fixed buffers are responsible for the acidity, a theoretical and experimental approach was developed for the determination of the concentration and pK of the fixed buffer constituted by the glycocalyx. The pH of the solution in the LIS was measured by ratiometric fluorescence microscopy while the buffer concentration or composition of the bathing solutions was altered. In addition, the divalent cation Sr^{2+} was added to the perfusion solutions to displace protons from the fixed buffer sites for the determination of the fixed buffer properties. We conclude that the LIS contain 3.7 mM of pK 6.2 fixed buffer and that this buffer is responsible for the acidic microenvironment in the LIS.

Key words: pH — Glycocalyx — Strontium — Microclimate — Fluorescence microscopy

Introduction

The lateral intercellular spaces (LIS) separating MDCK renal epithelial cells were reported to be more acidic by about 0.4 pH units than the bathing solutions (Chatton & Spring, 1994). Because a variety of inhibitors of acid extrusion from cells or a reduction in temperature failed to abolish this pH difference, the acidity of the LIS was attributed to the buffering effect of the negatively charged glycocalyx on the lateral membranes of the cells rather than to the cellular extrusion of acid or uptake of base (Chatton & Spring, 1994). An abundant glycocalyx fills the extracellular spaces of renal tubular epithelia (Weinstein et al., 1997) and the basolateral surfaces of cultured renal epithelial cells (Stow & Farquahar, 1987). It is a frequent current finding that many integral membrane proteins are heavily glycosylated. The pH within and near to the glycocalyx is predicted to be low because of electrostatic effects arising from the fixed negative charges of the sugar moieties and membrane phospholipid head groups (Schnitzer, 1988).

From an acid-base balance perspective, the LIS can be viewed as a compartment filled with two buffers — those fixed in the glycocalyx and the mobile buffers in the perfusion solution. The two buffer systems effectively compete for protons and the resultant pH depends on the balance between them. Mathematical models of such fixed/mobile buffer systems have been used to predict proton diffusion in the intracellular environment where fixed charges of cytoplasmic proteins constitute the immobile buffer and freely diffusible ionic buffers constitute the mobile fraction (Irving et al., 1990; Junge & McLauglin, 1987).

In the present study, we estimated the concentration and pK of the endogenous fixed buffer within the LIS of MDCK cells from measurements of the changes in LIS pH in response to a variety of perturbations. To this end, we adapted the models of fixed/mobile buffer systems to the LIS and used these models to calculate the buffering power of the glycocalyx.

Materials and Methods

Cell Culture

Low-resistance MDCK cells, passages 68–76 from the American Type Culture Collection (Rockville, MD), were cultured on permeable sup-

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ports (Anocell, Whatman, Clifton, NJ) that had been treated to reduce pore size as previously described (Chatton & Spring, 1994). Cells were grown for 13–43 days in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (Gibco, Grand Island, NY) and 2 mM glutamine without riboflavin, antibiotics and phenol red.

Experimental Solutions

The isotonic control experimental solution was (all in mM): 142 Na^+, 5.3 K^+, 1.8 Ca^{++}, 0.8 Mg^{++}, 137 Cl^-, 0.8 SO_4^{2-}, 14 HEPES, 5.6 glucose; the pH was adjusted to 7.4 at 37°C. The osmolarity of all solutions was 292–298 mOsm/kg. Increased HEPES concentration to 30 mM was made by reducing the Cl^- concentration accordingly; reduction in HEPES concentration to 5 mM was accompanied by a commensurate increase in Cl^- concentration. When the divalent cation Sr^{2+} was added, a corresponding reduction in Na^+ was made and the anion concentration was unchanged. Sufficient mannitol was added to maintain the osmolality at the control level.

Perfusion System

The monolayers were transferred to a closed chamber, placed on the microscope stage, and perfused on both apical and basal baths as described in Chatton and Spring (1994). The perfusion solution was switched rapidly by computer-controlled pinch valves.

Materials

The cell-impermeant fluorescent dye 2′,7′-bis (carboxyethyl)-5,6-carboxyfluorescein (BCECF, Molecular Probes, Eugene, OR) was used to determine LIS pH by excitation ratio imaging. BCECF was loaded into the LIS as previously described (Chatton & Spring, 1994). In brief, after the epithelium and its permeable support had been mounted in a bilateral perfusion chamber, 2 mM BCECF was added to the apical bath perfusate for a period of 1–3 min. During this period, BCECF diffused across the tight junctions and into the LIS. When the dye-containing solution was washed out, the LIS pH could be measured from the BCECF fluorescence for a period of 5–10 min before diffusional dye loss reduced the signal-to-noise to an unacceptable level. BCECF pulses were repeated several times in the course of an experiment.

Fluorescence microscopy

The experiments were performed on the stage of an upright microscope (Ortholux II, Leica, Deerfield, IL) equipped for differential interference contrast (DIC) and low light level fluorescence (Chatton & Spring, 1994). Epifluorescence illumination for excitation ratio imaging was achieved at 458 and 488 nm using an acousto-optical tunable filter (TEAF-.40.55, Brimrose, Baltimore, MD) attached to a multiline argon laser (532A, Melles Griot, Carlsbad, CA) as previously described (Chatton & Spring, 1994). The light source was connected to the microscope by means of a fused silica optical fiber (C Technologies, Verona, NJ). The fluorescence filter cube contained a 510 nm dichroic mirror and a 515 nm barrier filter (Omega Optical, Brattleboro, VT). The DIC illumination source was a 50W tungsten halogen lamp (Leica) equipped with a calcite prism polarizer, a selected 512 nm narrow band interference filter (Omega) and a liquid crystal retarder (Meadowlark Optics, Longmont, CO).

The monolayers were observed through a 100×/1.3 N.A. objective lens (Nikon, Melville, NY), using a microchannel plate intensifier (KS-1381, Video Scope, Sterling, VA) and video camera (VS-2000N, Video Scope). An 8-frame running average was used to reduce the noise level of the image (LKH 9000, Video Scope) which was stored on an optical memory disc recorder (OMDR, TQ-2028F, Panasonic, Newark, NJ) for later off-line analysis. The sequence of events (e.g., solution valves, intensifier gain, illumination shutters, stepper motor) during the experiment was controlled by a computer using a custom-made program. Pairs of images taken at the two excitation wavelengths were thresholded and the brightly fluorescent LIS segments selected for calculation of the ratio. An average ratio for all interspaces in the microscopic field of view (approximately 40 μm diameter) was calculated and compared to a calibration curve generated from standard pH solutions filling glass capillaries as previously described (Chatton & Spring, 1994).

Theory

Consider the LIS to be a compartment containing two types of buffer — a mobile buffer that comes into the LIS from the bathing solutions, and a fixed buffer created by proton binding to negatively charged groups on the glycocalyx. We assume that the concentration of the mobile buffer, $c1$, in the LIS equals its concentration in the bathing solution and that the dissociation constant of the mobile buffer, K_1, is also the same as that in the bathing solution. Similarly, the fixed buffer has an unknown concentration, $c2$, and dissociation constant, K_2.

Local chemical equilibrium is given by:

$$M^- + H^+ \rightleftharpoons MH,\ F^- + H^+ \rightleftharpoons FH \tag{1}$$

where M^- is the basic form of the mobile buffer and MH is the acidic form. A similar equilibrium describes the fixed buffer system and F^- denotes the basic form and FH the acidic form of the fixed buffer. At an equilibrium pH, H_1, the concentrations of both buffers are assumed to be constant in time and given by:

$$MH_1 + M_1 = c1,\ FH_1 + F_1 = c2 \tag{2}$$

Assuming local chemical equilibrium for both buffers yields

$$\frac{M_1 \cdot H_1}{MH_1} = K_1, \qquad \frac{F_1 \cdot H_1}{FH_1} = K_2 \tag{3}$$

Equations (2) and (3) fully describe the buffer systems, but contain only 5 known parameters — K_1, $c1$, H_1, MH_1, M_1 — and 4 unknowns — FH_1, F_1, K_1, $c2$. More information is required to obtain a solution. This was accomplished by perturbing the buffer system in three ways: (i) by changing the concentration, $c1$, of the mobile buffer; (ii) by the use of another mobile buffer with a different K_i, (iii) by the addition of the divalent cation Sr^{2+}.

It is assumed that the initial interaction of Sr^{2+} ions occurs with fixed charges of the glycocalyx (and cell membranes) and that Sr^{2+} ions occupy sites on the cell surface that would otherwise be available to H^+. We ignore possible entry of Sr^{2+} into the cells and interaction with intracellular buffers because of the slow time course expected for these events. Further, we assume that adsorption of Sr^{2+} to the fixed buffer can be characterized by the Langmuir isotherm

$$\Theta = \frac{K_A \cdot s}{(1 + K_A \cdot s)} \tag{4}$$

where K_A is the equilibrium constant for the process, s is the Sr^{2+} concentration, and Θ is the fraction of fixed buffer that is bound by Sr^{2+} and not available to H^+. At adsorption equilibrium, a fixed number of sites become incapable of binding protons. This is equivalent to a reduction in $c2$ to a new value $c3 = \Theta \cdot c2$. Such a change in fixed

buffer concentration must, in turn, lead to a change in the H^+ concentration in the LIS to a new value denoted as H_2 and new equilibria given by:

$$\frac{M_2 \cdot H_2}{MH_2} = K_1, \qquad \frac{F_2 \cdot H_2}{FH_2} = K_2 \tag{5}$$

The buffer concentrations at the new pH are now

$$MH_2 + M_2 = c1, \qquad FH_2 + F_2 = c2 - c3 = (1 - \Theta) \cdot c2 \tag{6}$$

The total amount of protons in the buffer system is virtually constant, determined by the buffer concentrations, and given by:

$$MH_2 + H_2 + FH_2 = MH_1 + H_1 + FH_1 \tag{7}$$

Expressing MH_2 and FH_2 from Eqs. (5) and (6) gives:

$$MH_2 = \frac{c1 \cdot H_2}{K_1 + H_2}, \qquad \mathrm{FH}_2 = \frac{(1 - \Theta) \cdot c2 \cdot H_2}{K_2 + H_2} \tag{8}$$

The variables MH_1 and FH_1 from Eqs. (2) and (3) may be expressed as:

$$MH_1 = \frac{c1 \cdot H_1}{K_1 + H_1}, \qquad FH_1 = \frac{c2 \cdot H_1}{K_2 + H_1} \tag{9}$$

Combining Eqs. (7), (8) and (9) yields:

$$\frac{c1 \cdot H_2}{K_1 + H_2} + \frac{(1 - \Theta) \cdot c2 \cdot H_2}{K_2 + H_2} + H_2 = \frac{c1 \cdot H_1}{K_1 + H_1} + \frac{c2 \cdot H_1}{K_2 + H_1} + H_1 \tag{10}$$

This expression contains three unknowns, K_2, c_2, and Θ. The third terms (H_1 and H_2) on both sides of Eq. (10) are at least three orders of magnitude smaller than the other two and can be neglected. Then $c2$ can be given by:

$$c2 = c1 \cdot \frac{\dfrac{H_2}{K_1 + H_2} - \dfrac{H_1}{K_1 + H_1}}{\dfrac{H_1}{K_2 + H_1} - \dfrac{(1 - \Theta) \cdot H_2}{K_2 + H_2}} \tag{11}$$

with $c2$ being positive when $H_2 > H_1$ (if K_2 is larger than K_1) and zero when $H_2 = H_1$, i.e., when there is no difference in pH in the two conditions. Experimentally, $c2$ and K_2, can be determined from Eq. (11) by measurement of the effects of Sr^{2+} addition on LIS pH when $c1$ is varied by changing mobile buffer concentration or when K_1 is changed by use of a different mobile buffer. It can be seen that for any given values of K_2 and $c2$ there is a critical value for Θ that is given by

$$\Theta_{cr} = 1 - \frac{H_1 (K_2 + H_2)}{H_2 (K_2 + H_1)} \tag{12}$$

Values of Θ greater than Θ_{cr} indicate that enough adsorption sites must be occupied (i.e, enough protons released) to produce an observable pH shift. In the limiting case when $\Theta = 1$, the second term in Eq. (10) is zero, and Sr^{2+} addition is the equivalent of adding a strong acid to the mobile buffer.

Data Analysis

Data are expressed as mean ± SEM. Differences were judged to be significant at the $P < 0.05$ level. Analysis of variance was performed on all groups, and the Bonferroni multiple comparison test was used to compute the significance of differences between groups.

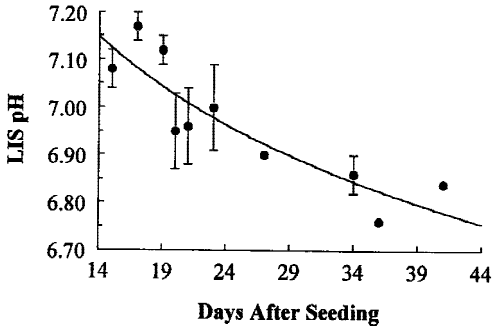

Fig. 1. LIS pH (mean ± SEM) as a function of the age of the cells in days after seeding on the permeable support. The perfusates in all cases were 14 mM HEPES of pH 7.4.

Results

Control LIS pH

Control LIS pH of MDCK cells grown on permeable supports and perfused with 14 mM HEPES buffered solutions was 6.99 ± 0.03 (mean ± SEM, $n = 27$), acidic to the pH 7.4 perfusates and in good agreement with the report by Chatton and Spring (1994). As shown in Fig.1, LIS control pH was a function of the number of days after seeding the cells on the supports. We observed that MDCK cells grow more slowly on permeable supports than on plastic dishes but exhibit stable morphology and geometry for several weeks after achieving confluence. Older cultures had more acidic LIS, presumably reflecting increased development of the glycocalyx. For reasons discussed below, all subsequent experiments were performed on cultures that were ~21 days old.

Varying the Mobile Buffer Concentration

As predicted by Eq. (11), varying the concentration of the mobile buffer changes the LIS equilibrium pH. Figure 2 shows the effect of varying the HEPES concentration from the control value of 14 mM to 5 or 30 mM. LIS pH in 5 mM HEPES fell significantly ($P < 0.01$) to 6.84 ± 0.03 (mean ± SEM, $n = 10$); in 30 mM HEPES, LIS pH rose significantly ($P < 0.05$) to 7.11 ± 0.03 (mean ± SEM, $n = 10$).

Varying the Fixed Buffer Concentration by Sr^{2+} Addition

When 25 mM of the divalent cation strontium was added to the perfusion solutions in place of sodium, the LIS was

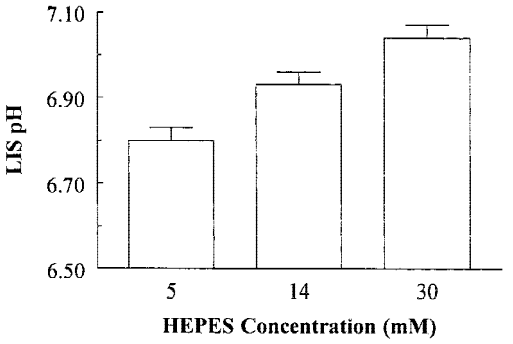

Fig. 2. Mean ± SEM LIS pH with 5, 14, and 30 mM HEPES concentrations. Both the 5 and 14 mM HEPES resulted in significantly different pH values from that in control (14 mM) HEPES.

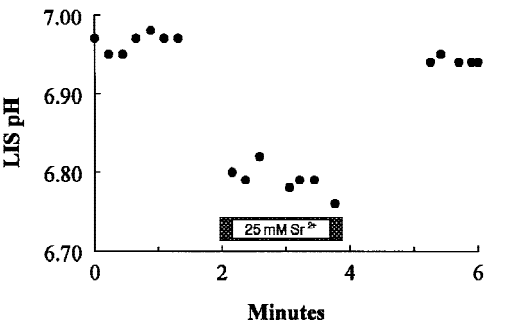

Fig. 3. Time course of a typical experiment with 25 mM Sr^{2+} addition in the presence of 14 mM HEPES.

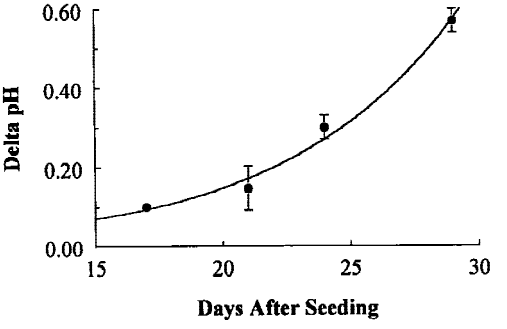

Fig. 4. Mean ± SEM magnitude of the Sr^{2+}-induced acidification (delta pH) in 14 mM HEPES compared to the number of days after seeding the cells on the permeable support.

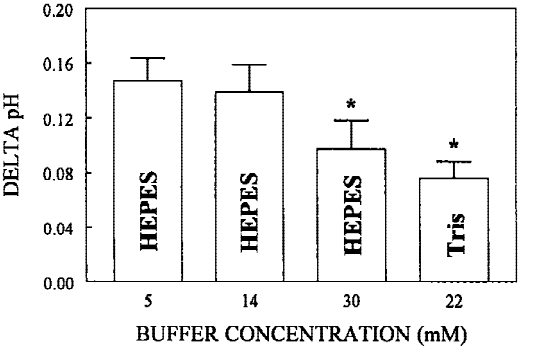

Fig. 5. Mean ± SEM delta pH caused by the addition of 25 mM Sr^{2+} in different concentrations of HEPES buffer or in Tris.

so rapidly acidified that the minimum pH was achieved by the first measurement after the solution change (Fig. 3). Cell size and shape did not change and the Sr^{2+} pulse could be repeated many times without any detectable effects on the appearance of the cells. As described by Eq. (4), Sr^{2+} binds to negative charges on the fixed buffer and displaces H^+ from these sites. The magnitude of the strontium-induced acidification also was a function of the age of the cell culture. As shown in Fig. 4, the magnitude of the pH decrease caused by Sr^{2+} increased markedly between 21 and 29 days after seeding of the cells on the permeable support. The striking dependence of the magnitude of the effect of Sr^{2+} on the age of the cultures after 21 days created potential difficulties in comparing results from different experiments. In addition, BCECF loading through the tight junctions into the LIS proved more difficult in the older cultures. Because of these concerns, all subsequent experiments were performed on cultures approximately 21 days after seeding.

As described by Eq. (11), the effect of Sr^{2+} addition on LIS pH depends on both the mobile buffer concentration, *c1*, and K_1, its equilibrium constant. Figure 5 compares the effect of 25 mM Sr^{2+} on LIS pH when the HEPES concentration was 5, 14, or 30 mM. Figure 5 shows that the addition of 25 mM Sr^{2+} results in a pH decrease of 0.139 ± 0.075 units ($n = 14$) in the presence of 14 mM HEPES. As predicted, increasing the concentration of the mobile buffer to 30 mM significantly diminished the magnitude of the pH decrease caused by Sr^{2+} addition.

Also shown in Fig. 5 are the results of Sr^{2+} addition experiments in the presence of 22 mM Tris, a concentration chosen to give approximately the same buffering capacity as 14 mM HEPES at pH 7.1. The pK_a of Tris at 37°C is 7.77, while the pK_a of HEPES at 37°C is 7.31. The pH decrease in the presence of Tris is 0.076 ± 0.041 units ($n = 11$), significantly less than that in HEPES ($P < 0.05$).

The pH decrease caused by Sr^{2+} addition should decrease at lower Sr^{2+} concentrations as Θ in Eq. (4) is dependent on the Sr^{2+} concentration. The acidification of the LIS in 14 mM HEPES caused by the addition of 10 mM Sr^{2+} was significantly ($P < 0.02$) diminished compared to that seen with 25 mM Sr^{2+}. The pH decrease with 10 mM Sr^{2+} was 0.079 ± 0.01 units ($n = 12$).

Estimation of Fixed Buffer pK

The Table shows the pK estimated for the fixed buffer from all of the Sr^{2+} addition experiments described

Table 1. Estimation of fixed buffer pK from Sr^{2+} addition experiments

Buffer (concentration)	LIS pH	pK of Fixed buffer	Number of exits.
HEPES (5 mM)	6.84 ± 0.03*	6.13 ± 0.07	10
HEPES (14 mM)	6.99 ± 0.03	6.27 ± 0.06	27
HEPES (30 mM)	7.11 ± 0.03*	6.53 ± 0.08	10
TRIS (22 mM)	6.86 ± 0.15	5.76 ± 0.08*	11
	Mean ± SEM	6.20 ± 0.05	58

* indicates significantly different ($P < 0.05$) from the 14 mM HEPES control. Fixed buffer pK was calculated from experiments involving Sr^{2+} addition as described in the text.

above. The 14 mM HEPES value in the Table incorporates the results from both the 10 and 25 mM Sr^{2+} experiments. Overall, the pK data are normally distributed and the average pK is 6.20 ± 0.05 ($n = 58$). It is not clear why the pK value calculated for the 22 mM Tris experiments is significantly lower than that in 14 mM HEPES.

Estimation of Θ and Fixed Buffer Concentration

An initial value for the fraction of fixed buffer sites bound by Sr^{2+}, Θ, was estimated from Eq. (4) using an equilibrium constant for Sr^{2+} binding of 233 M^{-1} (McLaughlin et al., 1981). Θ calculated in this fashion ranges from 0.7 in 10 mM Sr^{2+} to 0.85 for 25 mM Sr^{2+}. A more refined estimate was made subsequently using the fixed buffer pK value of 6.2. Assuming that the chemical composition of the fixed buffer, and hence its pK_a, are constant from one experiment to another, all of the experimental data can be fitted by varying *c2* and Θ to minimize the error in a plot of H_2 against H_1. The best fit for the 25 mM Sr^{2+} experiments in HEPES occurs when $\Theta = 0.9$ and the mean ± SEM fixed buffer concentration, *c2*, is 3.7 ± 0.23 mM.

Discussion

In a previous study (Chatton & Spring, 1994), it was shown that the low pH of the LIS of MDCK cells on permeable supports was not affected by inhibitors of acid extrusion from the adjacent cells. In the presence of 14 mM HEPES, LIS and cell pH are both about 7.0 (Chatton & Spring, 1994) so the total quantity of free protons in each compartment would be directly proportional to their respective volumes. The LIS compartment volume is about 5% of the intracellular one (Kovbasnjuk et al., 1995). Maintenance of the pH in this smaller compartment should require a correspondingly lower buffer concentration. In the present study, we determined the concentration of fixed buffer in the LIS to be 3.7 mM, about 5–10% of that estimated for the typical intracellular compartment (Roos & Boron, 1981).

We used brief pulses of the divalent cation strontium to perturb the binding of protons to the glycocalyx without disturbing the adjacent cells. In the analysis of our results, it was tacitly assumed that the effect of Sr^{2+} addition was due solely to interaction with the paracellular fixed buffer. Sr^{2+} has been reported to compete with Ca^{2+} for entry into erythrocytes (Raess & Keenan, 1996) as well as to slowly hyperpolarize peritoneal macrophages by activating K^+ channels (Araujo, Persechini & Oliviera-Castro, 1986). In addition, Sr^{2+} entry into MDCK cells would be expected to displace H^+ from intracellular fixed buffers and acidify the cell interior. Acid extrusion across the basolateral cell membranes could then acidify the LIS. These effects should be relatively slow compared to the LIS pH changes observed in the present study, and we conclude that they are not responsible for the initial step change in LIS pH observed upon the addition of Sr^{2+}.

Since the LIS is essentially an open compartment at the basal end, maintenance of a lower pH would be aided by any restrictions to proton diffusion in the LIS. In a previous study, an attempt was made to estimate a minimum value for the diffusion coefficient for protons within the LIS. No reduction in diffusivity could be detected although the temporal sensitivity of the approach was only adequate to detect reductions in diffusivity of 100-fold or more (Chatton & Spring, 1994). Fixed charges that bind protons would be expected to reduce proton mobility within the charged layer that constitutes the buffer. The experiments in the present study show that the acidic region between MDCK cells arises because of buffering by fixed negative charges on the surface of the lateral membranes forming the LIS. The glycocalyx is the most likely source of these fixed negative charges, although phospholipid head groups could also be significant components of the fixed buffer.

Because the pH in the LIS is substantially lower than that in the bulk solution, the affinity of compounds for binding sites and the kinetics of processes taking place at the basolateral cell membrane may be significantly affected. The acidic microclimate of small intestinal epithelia has long been invoked as an important factor in the partitioning of weak electrolytes across the epithelium (Shiau et al., 1985). Similar concerns have been expressed about the influence of a low near-membrane pH on the diffusion of formic acid across the apical membranes of proximal tubule cells (Aronson & Giebisch, 1997). A low pH near the cell membrane is not a surprising finding in light of the decades of previous reports on the influence of the negative charges of membrane phospholipids and the glycocalyx on the electrostatics in the boundary layer near the membrane surface (Schnitzer, 1988). To our knowledge, our study is the

first to estimate the concentration and pK of the fixed buffer constituted by the surface of the membranes of an epithelium.

It seems evident that the extracellular spaces surrounding the basolateral surfaces of MDCK cells, and presumably most other epithelia, are not simple extensions of the bathing solutions, but constitute an altered, acidic environment. In addition to the generation of a low surface pH, the electrostatic effects of the glycocalyx may influence cell-cell attachments, cell shape and proliferation. An understanding of the consequences of the fixed charges present on the membrane surfaces will require a detailed examination of the solute concentrations in the boundary layer adjacent to the cell membrane to ascertain whether physiologically relevant ion concentration gradients exist within the glycocalyx.

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