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Effect of extracellular pH on cytoplasmic pH and mechanism of pH regulation in cultured bovine corneal endothelium: Possible importance in drug transport studies

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Summary

Bovine corneal endothelial cells were grown in culture and the effect of extracellular pH on cytoplasmic pH of the cells was examined using a cytoplasmic-trapped fluorescent dye: bis-carboxyethyl-carboxyfluorescein. The results indicate that these two pH values are different and, if these results are indicative of what occurs in intact tissue, the transport of weak acids and bases can be greatly affected when traversing through viable cells. Furthermore, a reversible Na^+/H^+ exchanger, which is sensitive to amiloride, has been identified in the regulation of cytoplasmic pH of cultured bovine corneal endothelium.

Introduction

Understanding the relationship between pH and drug absorption across viable biological membranes is important in the process of optimizing drug delivery. Typically, in an ex vivo permeability experiment, the tissue of interest is isolated and placed in contact on both sides with a buffer of varying pH (e.g. Schoenwald et al., 1983; Dowty, 1988; Mitra et al., 1988). Permeability coefficients are then calculated for the drug vs. bathing buffer pH but without a clear understanding of the pH gradient inside the tissue. Several important questions can be raised relative to this experimental design and interpretation of results. How does the extracellular pH (pH_e) affect the cytoplasmic pH (pH_c) of viable cells? By what mechanism do cells regulate pH_c? And what ramification does this have on weak acids and bases traversing through these cells? These questions are addressed for cultured bovine corneal endothelium (BCE) in the present study using a fluorescent, pH-sensitive dye 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF). It is clear that the pH gradient in intact tissue may be different than in cultured cells. However, since most cells have the ability to regulate pH_c, this work will serve as a first approximation to what is happening in situ.

BCECF (see Fig. 1) has been shown to be a

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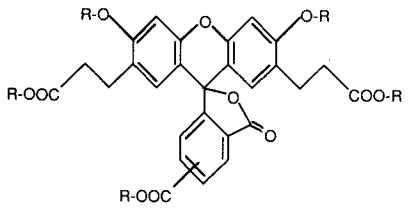

Fig. 1. Structure of 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and the acetoxymethyl ester form of the dye (BCECF/AM).

useful probe for continuous measurements of pH_c in a variety of cells (Rink et al., 1982; Paradiso et al., 1984; Graber et al., 1986; Berk et al., 1987; Henderson et al., 1988; Kotyk et al., 1989; Brion et al., 1990). The fluorescent dye, with a pK_a of 6.98, exhibits a nearly linear relationship between pH and fluorescence, at least between pH 6.4 and 7.4, and is more linear than other pH dyes (Rink et al., 1982). The pentaacetoxymethyl ester of BCECF (BCECF/AM; see Fig. 1) is membrane permeable, whereupon entering the cell, it forms the relatively membrane impermeable BCECF via cleavage by cytoplasmic esterases (Rotman et al., 1966; Thomas et al., 1979, 1982; Rink et al., 1982; Thomas, 1986; Kolber et al., 1988; Kotyk et al., 1989). BCECF is a cytoplasmic-trapped dye which monitors pH_c and not that of other cellular compartments (Rink et al., 1982; Paradiso et al., 1984). It should be noted that the pH_c which is measured by BCECF is an average pH value and pH gradients may exist inside the cell.

Materials and Methods

Cell culture

Bovine corneal endothelial cells were generously provided by Jacqueline Tassin (INSERM, Paris, France). These cells were extracted by the procedure of Arruti et al. (1982). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (L-glutamine from Sigma, St. Louis, MO; all other products from Flow Laboratories, Irvine, U.K.) seeded into Nunclon® Delta culture flasks (Inter Med Nonc, Denmark). Cells were incubated at 37°C and gassed with 5% CO_2/95% air. Culture medium was changed every 3 days. Subcultures were made with 0.05% trypsin/0.02% EDTA in modified Puck's saline A (Gibco BRL, Paris, France) and passaged at a split ratio of 1:2. Cultures became confluent after a period of 3–5 days at this ratio and experiments were performed with 3rd–5th passage cells only, waiting at least 1 week after subculture.

Measurement of pH_c using BCECF

Cells were detached using 0.05% trypsin/0.02% EDTA, followed by addition of serum and modified Hank's balanced salt solution (Flow Laboratories) supplemented with 0.5 mM Ca^{2+} and 1.0 mM Mg^{2+}. Cells were centrifuged (1200 rpm, 7 min), resuspended in Hank's, and pelleted again. Cells were resuspended in Hank's to give a cell concentration of approx. 2×10^6/ml, and an aliquot was removed for determination of cell autofluorescence. The cell suspension was incubated with 3 μM BCECF/AM (Calbiochem, La Jolla, CA) for approx. 30 min at 37°C (±1°C). Cells were pelleted and resuspended in Hank's, repelleted, and suspended in 'Na-buffer' (i.e. Na-buffer is defined as consisting of 130 mM NaCl, 1.5 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, and 20 mM Hepes; pH 7.4) which is isoosmotic with biological fluids. The pH was adjusted with minute amounts of concentrated HCl or NaOH which did not change significantly the osmolarity of the solution. The cells were stored at room temperature in 1 ml aliquots in microcentrifuge tubes. Prior to fluorescence measurement, cells were warmed to 37°C for 5 min, pelleted, suspended in 2 ml freshly oxygenated buffer ($\approx 1 \times 10^6$ cells/ml), and placed in a quartz cuvette containing a magnetic stir bar. In pH_e vs pH_c experiments, Na-buffer at pH values of 6.2, 6.8, 7.4, and 8.0 was used for final cell resuspension. Evidence for Na^+/H^+ exchange in BCE was determined using nigericin, amiloride, ouabain, and ammonium (all chemicals from Sigma) in the presence and absence of extracellular Na^+ (details of experiments are included in Results and Discussion). A 'choline-buffer' (i.e. isoosmotic re-

placement in Na-buffer of 130 mM NaCl with 100 mM choline Cl; all other constituents were the same) was used to study the lack of Na^+ in the bathing solution. Cells showed greater than 96% trypan blue exclusion with this protocol.

Measurements of fluorescence were made with a Shimadzu RF-5000 spectrofluorophotometer (Kyoto, Japan) equipped with magnetic stirring apparatus and thermostated cuvette holder. Excitation and emission wavelength maxima were 506 and 526 nm, respectively, and bandwidth was 1.5 nm for both excitation and emission. Interference due to cell autofluorescence was, at least, less than 100 × the total signal of BCECF-loaded cells and did not change over the course of the experiment. Leakage rate of BCECF from cells was less than 2% over 10 min, estimated by initial and final dye in the supernatant. BCECF incorporated into cells appears to behave as dye in free solution exhibiting in both cases an emission maximum of 526 nm. Moreover, reproducible pH_c vs pH_e results were obtained with cells containing differing amounts of incorporated BCECF (i.e. 100 × –500 × background).

At the completion of each individual experiment, a calibration curve of fluorescence vs pH was constructed by measuring fluorescence and pH after solubilization of the cells with 30 μM digitonin and after each subsequent addition of small amounts of 330 mM 2-[*N*-morpholino]-ethanesulfonic (Mes) acid or base. The fluorescence calibration points enveloped the fluorescence measured during an experiment so that no data was obtained from extrapolated points.

Results and Discussion

Cell cultures

The spectrofluorophotometric technique used requires a large number of cells for measurement, much less than can be collected from a single eye. Therefore, for reasons of processing time, BCE was grown in culture. These cells closely resemble the parent, native endothelium with respect to cell shape and organelles and basement membrane formation (MacCallum et al., 1982). Moreover, metabolic pathways have been shown to be similar in freshly extracted and cultured BCE (Gerritsen et al., 1989). Cultured human corneal endothelial cells have also been used successfully for transplantation onto human corneas, for studying repair of damaged endothelium in situ, and for in vitro studies of cell growth regulation (Pistov et al., 1988). To this end, even though an incomplete characterization of cultured vs native cells exists, cultured cells represent a good first approximation to what is happening in situ.

pH_c vs pH_e

After cells were resuspended in Na-buffer at various pH values, fluorescence signals reached steady state very rapidly (i.e. before the cuvette could be placed in the spectrofluorophotometer which required several seconds) and this signal was constant for the duration of the experiment (i.e. ≤ 10 min). Fig. 2 shows that the pH_c is different from the pH of the bathing buffer. Interestingly, similar results were obtained from cultured rabbit buccal epithelial cells (unpublished data) which indicates that the corneal epithelium, as well as other cells, could possibly behave in a corresponding fashion. Apparently, there appear

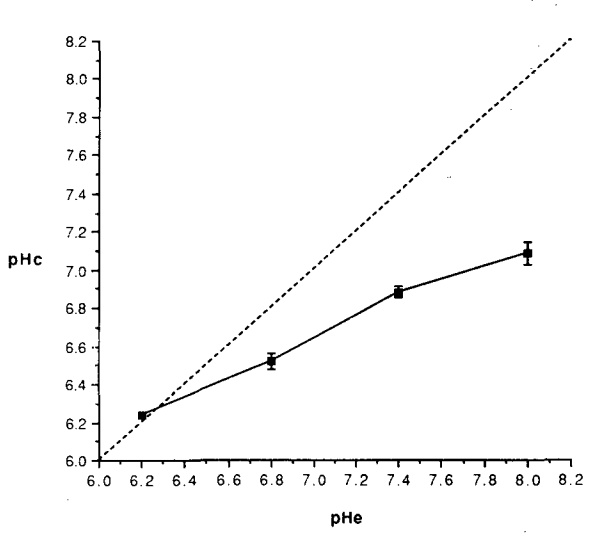

Fig. 2. Cytoplasmic pH (pH_c) in response to extracellular pH (pH_e) of cultured bovine corneal endothelial cells using the fluorescent dye BCECF. Each point is the average of four experiments where error bars are standard error of the mean. The segmented line represents a hypothetical situation where pH_e is equal to pH_c.

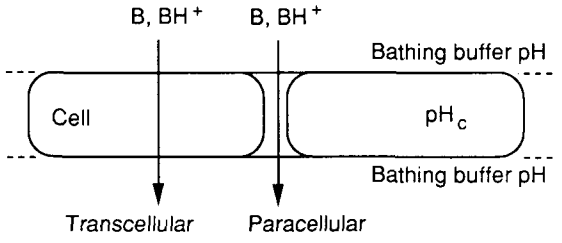

Fig. 3. Schematic representation of the species of a weak base (i.e. B and BH^+) traversing a viable single-cell endothelium via transcellular and paracellular routes.

to be similar mechanisms of pH regulation in many different epithelial cells which have been examined by others (Aronson, 1985; Moolenaar, 1986; Frelin et al., 1988; Grinstein et al., 1989) and will be discussed in a subsequent section.

The theoretical ramifications of these results are best illustrated with the following example. A weak base with a pK_a of 6.9 crosses 1 cm^2 of single-cell endothelium by simple diffusion in an ex vivo permeability experiment where the bathing buffer pH on both sides of the monolayer is the same (see Fig. 3). The following assumptions will be applied to the example diffusion study: (1) endothelium remains viable in that cells are able to regulate pH; (2) results from pH_c vs pH_e data are representative of what is seen in the native corneal endothelium; and (3) intercellular pH is the same as bathing buffer pH. At this point, it is necessary to derive an equation for total flux which includes the pH effect of the environment.

The drug's behavior in aqueous solution can be represented by the following equilibrium:

$$BH^+ \leftrightarrow B + H^+$$

The relative concentrations of ionized and unionized forms are governed by:

$$K_a = ([S_B][S_{H^+}]/[S_{BH^+}]) \quad (1)$$

where [S] is the concentration of each species and K_a is the acid dissociation constant. It is assumed, in this example, that the flux of each species (J) crossing a single viable cell layer by simple diffusion at steady state is determined by:

$$J_B = D_B K_B C_B/h = P_B C_B \quad (2)$$

$$J_{BH^+} = D_{BH^+} K_{BH^+} C_{BH^+}/h = P_{BH^+} C_{BH^+} \quad (3)$$

where D, K, P, and C are the diffusion coefficient, partition coefficient, permeability coefficient, and donor concentration of each species, respectively, and h is the thickness of the single cell layer. Note that the electric field across the monolayer and activity of each species are assumed constant in using Eqns 2 and 3.

Total flux (J^o) can then be written as the sum of the paracellular and transcellular fluxes (J^P and J^T respectively):

$$J^o = J^P + J^T \quad (4)$$

$$J^o = P_B^P C_B + P_{BH^+}^P C_{BH^+} + P_B^T C_B + P_{BH^+}^T C_{BH^+} \quad (5)$$

where P^T and P^P are the apparent permeability coefficients of each species transported via the transcellular and paracellular routes, respectively.

The concentration of each species present relative to pH can be described by:

$$C_B = X_B C^o \quad (6)$$

$$C_{BH^+} = X_{BH^+} C^o \quad (7)$$

where C^o is total concentration of all species and X is the mole fraction of each species present calculated from the Henderson-Hasselbalch equation. Hence, Eqn 5 can be rewritten as:

$$J^o = C^o\left(X_B^P P_B^P + X_{BH^+}^P P_{BH^+}^P + X_B^T P_B^T + X_{BH^+}^T P_{BH^+}^T\right) \quad (8)$$

where X^P and X^T are the mole fractions of each species in the paracellular and transcellular routes, respectively.

From the hypothetical flux data shown in Table 1, Eqn 8 can now be used to formulate a system of four linear equations to compute permeability coefficients of the various species which can be solved with matrix arithmetic. The calculated values of P_B^P, $P_{BH^+}^P$, P_B^T, and $P_{BH^+}^T$ for this system are 1.54×10^{-5}, 5.37×10^{-6}, 1.05×10^{-4}, and 5.63×10^{-6} cm/s, respectively. To this end, knowledge of internal tissue pH leads to a greater level of understanding of the permeability of dissociable species through conducting routes. In-

TABLE 1

Theoretical flux data for a weak base (pK_a 6.9; donor concentration: 10^{-5} mol cm^{-3}) traversing a cell monolayer assuming that the pH_c varies with bathing buffer pH (pH_b) as in Fig. 2

pH_b	pH_c	J^o (mol cm^{-2} s^{-1}) ($\times 10^{10}$)	X_B^P	$X_{BH^+}^P$	X_B^T	$X_{BH^+}^T$
8.0	7.1	8.0947	0.926	0.074	0.613	0.387
7.4	6.9	6.8100	0.760	0.240	0.500	0.500
6.8	6.5	4.3645	0.443	0.557	0.285	0.715
6.2	6.2	2.9094	0.166	0.834	0.166	0.834

deed, the intracellular and intercellular pH gradients will presumably be more complex in a multiple-cell layer system and, consequently, flux equations will also be much more elaborate than those used in the above example.

For the sake of simplicity, this example was based on a number of assumptions, the most important being that the results from pH_c vs pH_e data of cultured cells are indicative of intact tissue. It is understood that differences exist between cells in suspension and those that are in direct communication with their neighbors. However, because most cells are capable of regulating pH_c, this phenomenon is an important consideration when performing an ex vivo viable tissue permeability experiment with a weak acid or base.

Mechanism of pH_c regulation

H^+ is distributed across the plasma membrane of most cells such that the internal pH is higher than predicted if H^+ were in passive equilibrium (Grinstein et al., 1989). Hence, these cells possess a type of mechanism to extrude H^+, thus maintaining the internal pH at this higher than equilibrium value. The Na^+/H^+ exchanger has been implicated in internal pH regulation, and appears to be present in the plasma membrane of all types of vertebrate cells (Aronson, 1985; Moolenaar, 1986; Frelin et al., 1988; Grinstein et al., 1989) including epithelial cells from the renal tubule, intestine, and the urinary and gall bladders. In terms of the mechanics of the transporter, Na^+ can drive H^+ out of or into the cell depending on the concentration gradient of Na^+.

Jentsch et al. (1985) have already shown some evidence for Na^+/H^+ exchange activity in cultured BCE using ^{22}Na uptake studies. The following three studies using BCECF, a preferred technique over uptake studies, indicate the presence of a reversible Na^+/H^+ exchanger, which is sensitive to amiloride, in cultured BCE.

Effects of nigericin with and without amiloride on pH_c of BCE in choline-buffer Cells were resuspended in choline-buffer to eliminate the possibility of Na^+/H^+ exchange. Nigericin (5 μg/ml), a K^+/H^+ ionophore, was added to acidify the cells by the driving outward flux of K^+ and subsequent inward flux of H^+. Albumin (5 mg/ml) was then added to neutralize nigericin followed by addition of 100 mM NaCl. In effect, Na^+ is now available to exchange with H^+, and if the Na^+/H^+ exchanger exists, cells will regulate their pH_c back toward basal levels. Indeed, this is the case in Fig. 4A indicating evidence for an Na^+/H^+ exchanger in BCE. Note that the final plateau pH_c is slightly below the original basal value possibly due to

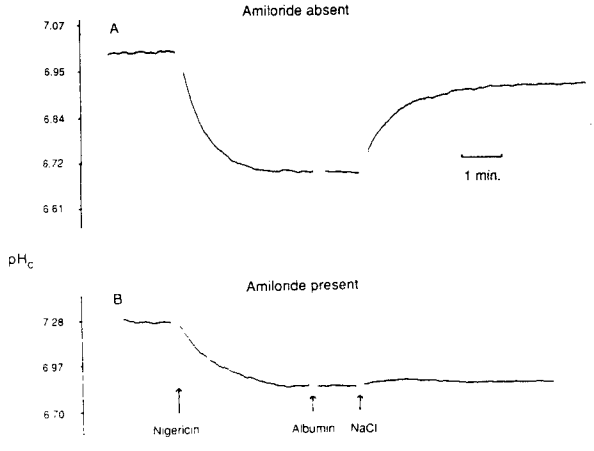

Fig. 4. Effects of nigericin with and without amiloride on pH_c of bovine corneal endothelium. (A) Cells were washed once and resuspended in choline-buffer. Nigericin (5 μg/ml) was added (arrow) to acid-load cells and then 5 mg/ml bovine serum albumin was added to bind and to inactivate nigericin. Cells regulated back toward basal pH_c levels only after addition of 100 mM NaCl (arrow). (B) The procedure was identical to (A) however, 1 mM amiloride was present throughout the experiment. After 100 mM NaCl was added, cells could not regulate pH_c because of blockage of Na^+/H^+ exchange by amiloride. All traces represent one of at least four identical experiments.

some residual nigericin activity. Amiloride (1 mM), an inhibitor of Na^+/H^+ exchange, can prevent cells from regulating pH_c even in the presence of Na^+ as seen in Fig. 4B. Note that because amiloride is an organic base, the starting baseline pH in trace B is more alkaline than that of trace A (this is similar to the effect by ammonium chloride in Fig. 6, trace A, which is discussed below).

Effects of Na^+ and Na^+-free buffers on pH_c of ouabain-treated BCE Normal Na^+/K^+ ATPase activity in BCE was blocked with 0.1 mM ouabain for 45 min increasing intracellular Na^+ concentration. If Na^+/H^+ exchange exists, then, when cells are resuspended in choline-buffer, Na^+ will exchange out of the cells down its concentration

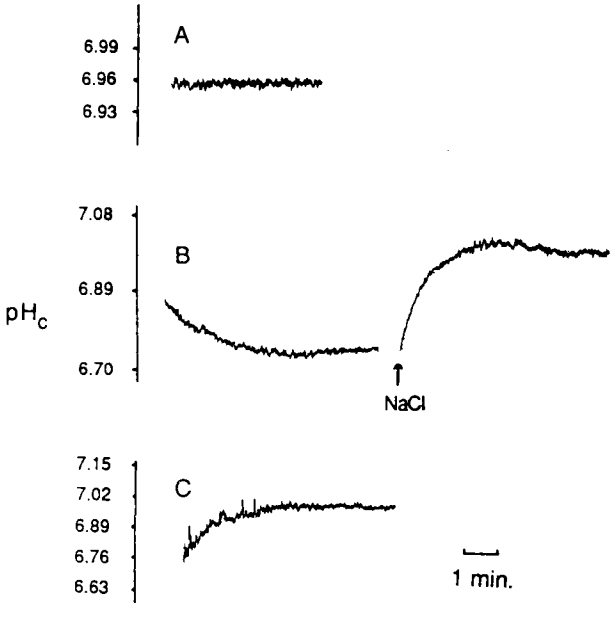

Fig. 5. Effects of Na^+ and Na^+-free buffer on pH_c in ouabain-treated bovine corneal endothelium. Cells were treated with 0.1 mM ouabain in Na-buffer for 45 min. Cells were then washed once in the same or choline-buffer before suspension in final test buffer: (A) cells were resuspended in Na-buffer containing ouabain and the pH_c remained at 6.96; (B) cells were resuspended in choline-buffer containing ouabain and the pH_c decreased to about 6.75 where this acidification was reversed when 100 mM NaCl was added to the cell suspension; (C) cells were washed in choline-buffer containing ouabain for 5 min and then resuspended in Na-buffer and cells became alkaline at a rate similar, but not identical, to that observed in (B) where the cells were in a hypertonic environment. All traces represent one of at least four identical experiments.

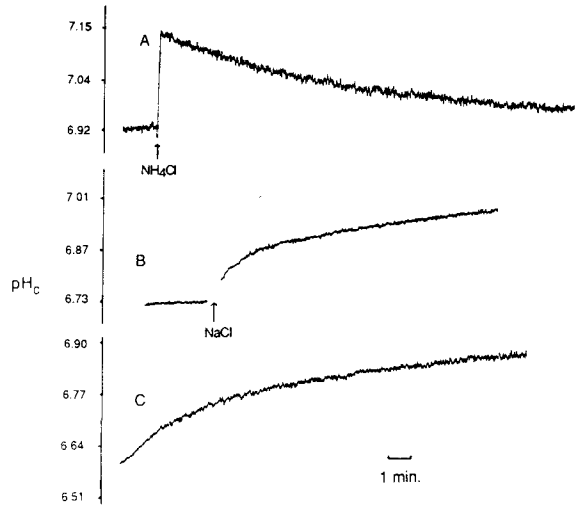

Fig. 6. Effects of NH_4Cl on pH_c of bovine corneal endothelium in Na- and choline-buffers. (A) Cells were suspended in Na-buffer and 20 mM NH_4Cl was added (arrow) where pH_c increased to about 7.15 and subsequently relaxed back toward basal pH_c. (B) Cells were incubated in Na-buffer containing 20 mM NH_4Cl for 4 min, washed once in choline-buffer and then resuspended in choline-buffer. Cells did not regulate pH_c until 100 mM NaCl was added (arrow). (C) Cells were suspended in Na-buffer containing 20 mM NH_4Cl for 4 min, washed once in choline-buffer and then resuspended in Na-buffer (isoosmotic conditions) where cells immediately regulated pH_c back toward basal levels at a similar rate seen in (B). All traces represent one of at least four identical experiments.

gradient thus acidifying the cells by the opposite inward exchange of H^+. Ion exchange should be reversible with addition of extracellular Na^+ thereby changing the Na^+ chemical potential. This phenomenon is evident from Fig. 5B showing further evidence for Na^+/H^+ exchange activity and, moreover, that this transport system is reversible in BCE. It should be noted that with the addition of NaCl in Fig. 5B, cells were exposed to a hypertonic solution. Hence, to test whether hypertonicity may stimulate Na^+/H^+ exchangers (Green et al., 1988) which are normally inactive, cells were resuspended in Na-buffer instead of adding NaCl. Fig. 5C shows that pH_c was immediately regulated back toward basal levels indicating minimal, if any, hypertonic activation of Na^+/H^+ exchange.

Effects of NH_4Cl on pH_c of BCE in Na- and choline-buffers When 20 mM NH_4Cl is added to BCE in Na-buffer, cells alkanalize with subse-

quent regulation back toward basal pH_c (Fig. 6A). A Cl^-/HCO_3^- exchanger appears to be responsible for the reacidification after addition of NH_4Cl (Jentsch et al., 1985; Reinertsen et al., 1988). If BCE are exposed to NH_4Cl for 4 min in Na-buffer and then resuspended in NH_4-free choline buffer, cells acidify due to the redistribution of ammonium. Moreover, HCO_3^- levels inside the cell may be low at this point because of possible earlier exchange with Cl^-. Cells should then realkanalize with the addition of NaCl in the presence of Na^+/H^+ exchange as observed in Fig. 6B. As in the study with ouabain, hypertonicity appeared to play a minimal, in any, role in activation of Na^+/H^+ exchange (Fig. 6C).

Conclusion

The results here suggest that the pH_c can be significantly different from the pH_e of cultured BCE. The hypothetical implications of these results have been addressed for a weak base traversing a viable single-cell layer. It is clear that this work was performed with cultured cells and uncertainties exist with respect to native cells. However, since most cells appear to have some sort of internal pH regulation mechanisms, this work will serve as a prelude to pH_e effects on pH_c of cells in situ. To this end, knowledge of the effect of pH_e on pH_c will facilitate in the interpretations of a weak acid or base traversing cellular tissue.

A reversible Na^+/H^+ exchanger, which is sensitive to amiloride, has also been implicated in pH_c regulation of cultured BCE. However, it appears other pumps are involved in the regulation of pH_c as well (e.g. Cl/HCO_3^- exchanger) in BCE.

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