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Cytoplasmic pH in isolated rat enterocytes. Role of Na^+/H^+ exchanger

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The cytoplasmic pH (pH_i) was determined in isolated rat intestinal cells with four methods. The pH_i of cells in physiological saline buffered with Hepes (pH 7.3) at 37°C was close to 7.0. The most reliable method, using the fluorescent pH indicator 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF), furnished a mean value of 7.03 ± 0.05 ($n = 42$). The buffering capacity of intestinal cells determined with this fluorescent indicator was 62 ± 5 mmol · l^{-1} · pH^{-1}. The mechanism governing the control of cytoplasmic pH was also investigated with BCECF, varying the Na^+ concentration inside and outside the cells. When intestinal cells were suspended in a sodium-free medium in the presence or absence of ouabain, they became acidified. The process was reversed when Na^+ was added to the incubation medium. An identical phenomenon occurred when the cells were artificially acidified with NH_4Cl. Additional experiments led to the conclusion that isolated rat intestinal cells have an Na^+/H^+ exchanger independent of Cl^- and inhibited by amiloride. This exchanger plays an important but not exclusive role in the control of pH_i. The presence of other exchangers and the high buffering power of the cells explains the high stability of pH_i noted in this study.

Introduction

The H^+ concentration plays a major role in many cell processes [1]. The determination of the normal cytoplasmic pH (pH_i) is thus indispensable for the study of cell functions. Numerous data have been published on the pH_i of various mammalian cells (for a review see Ref. 2); in contrast, there have been few studies of the cytosolic pH of isolated enterocytes [3,4]. This would appear to be fundamental for the further understanding of intestinal absorption mechanisms [5]. The pH_i was determined by four methods: (a) the external pH (pH_o), at which disruption of the intestinal cell membranes by digitonin produced no shift in the pH_o was taken as a measure of pH_i; (b) the equilibrium distribution of the weak acid, 5,5-dimethyloxazolidine-2,4-dione (DMO), was used to calculate intracellular pH; (c) after rapidly centrifuging intestinal cells, they were lysed and the pH was measured directly; (d) the fluorescent pH indicator, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was used in the form of membrane-permeant ester. BCECF released by cell esterases exhibited a linear relation between pH and fluorescence, at least between pH 6.4 and 7.4 [6,7].

A large body of recent work has implicated Na^+/H^+ exchange in the mechanism of pH_i control in a number of cell types (for a review see Ref. 8). The existence of an Na^+/H^+ exchanger has been demonstrated in intestinal cell mem-

Abbreviations: BCECF, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein; DMO, 5,5-dimethyloxazolidine-2,4-dione; DMSO, dimethyl sulfoxide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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branes [9–11], but only one report exists for rabbit isolated intestinal cells [4] and none for rat enterocytes. The purpose of the present work was to investigate how isolated rat enterocytes regulate their pH_i. The experimental basis was to monitor pH_i during conditions under which Na^+ concentration gradient across the cell membranes was oriented outward ($[Na^+]_i > [Na^+]_o$) or inward ($[Na^+]_i < [Na^+]_o$). All solutions used were depleted of HCO_3^- and some also lacked Cl^-, in order to eliminate any possible contribution of a Cl^-/OH^- (HCO_3^-) exchanger [12].

Materials and Methods

Chemicals and solutions

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. Stock solutions of digitonin (10 mg/ml) in DMSO and monensin (0.8 mg/ml) in DMSO/ethanol (3 : 1, v/v) were stored at 4° C. BCECF/AM (acetoxymethyl ester) was from Calbiochem. Stock solution of BCECF/AM (0.1 mM) in DMSO was stored at −20° C. [*carboxyl*-^{14}C]Dextran (1.24 mCi/g), ^3H_2O (0.25 mCi/g) were from New England Nuclear. [^{14}C]DMO (50 mCi/mmol) was from Amersham. The scintillation liquid (RIA-LUMA) was purchased from Lumac.

Hanks' medium was composed of 25 mM Hepes (pH 7.3), 137 mM NaCl, 5 mM KCl, 0.6 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 5 mM glucose. In choline chloride solution, we replaced NaCl with choline chloride and in choline-CH_3SO_3 solution the Cl^- was replaced by $CH_3SO_3^-$ or SO_4^{2-}. A stock solution of choline-CH_3SO_3 (1 mM, pH 7.0) was prepared by mixing 2 M CH_3SO_3H with an equimolar concentration of choline hydroxide and this was stored at 4° C. K^+ solution comprised 140 mM KCl, 20 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.5 mM Hepes or Mes. pH was adjusted to the required value (6.4–7.4) with addition of 0.1 M HCl or NaOH.

Isolation of intestinal cells

The methods of Weiser [13] and of Hegazy et al. [14] were used to isolate intestinal cells from Wistar rats. This technique has been published elsewhere [15]. Isolated cells were suspended in Hanks' medium (1 mg cellular protein/ml). Cell viability was checked with the Trypan blue dye exclusion method [16]. Protein was assayed with the method of Lowry et al. [17].

pH_i measurement

(a) Digitonin null-point. Cells were first incubated in Hanks' medium (pH 7.3) at 37° C for at least 45 min. Aliquots were then washed and resuspended in the K^+ lightly buffered solution to a concentration of near 1 mg cellular protein/ml. With the suspension continually stirred, external pH (pH_o) was monitored and recorded on a pen recorder, and adjusted to the required value with addition of 0.1 M HCl or NaOH. Digitonin was then added to give a final concentration of 70 μM. The maximum pH shift was noted. The cells were in the K^+ solution for only 2–3 min.

(b) DMO method. Steady-state intracellular pH was determinated by measuring the distribution of the weak acid, [^{14}C]DMO. After 15 min preincubation period in Hanks' medium (pH 7.3, 37° C), the cell suspension (1 mg cellular protein/ml) was loaded with [^{14}C]DMO (1 μCi/ml) and ^3H_2O (2.5 μCi/ml). At 25 min, aliquots (200 μl) of the suspension were removed and the cells were separated from the medium by the rapid sampling silicone oil method [18]. Intracellular radioactivity was determinated with an LKB-1215 Rackbeta liquid scintillation spectrometer under conditions for determining double-labeled samples. The extracellular and intracellular spaces were determined by replacing [^{14}C]DMO with [*carboxyl*-^{14}C]dextran (1 μCi/ml) and used to determine the DMO distribution. Intracellular pH was calculated according to the equation of Waddel and Butler [19]:

$$pH_i = pK_a + \log\left[\frac{[DMO]_i}{[DMO]_o}(1 + 10^{pH_o - pK_a}) - 1\right]$$

The value used in these experiments for the pK_a of DMO was 6.28.

(c) Cell lysate. About 0.5 ml of packed cells were centrifuged from the Hanks' medium, rapidly washed in lightly buffered K^+ solution, and then pelleted at $1500 \times g$. The supernatant was removed and the cells were homogenized using a Potter homogenizer with a Teflon pestle, with eight strokes at 2500 rpm. The pH electrode was

immersed in the disrupted cells and the pH recorded.

(d) Trapped fluorescent indicator. Isolated cells were incubated with BCECF/AM (2 μM) for 45 min at 37°C. The impermeant BCECF is generated in situ by the action of cytoplasmic esterases. The cells were then centrifuged and resuspended in Hanks' medium to give a stock suspension. As required, 1 ml of this stock was briefly centrifuged at $150 \times g$, washed twice with Hanks' medium and resuspended in 2 ml medium in a fluorescence cuvette. Thus any leaked dye was removed just before experimental observation. Under these conditions, the leakage rate of the dye from cells, estimated by initial and final dye in the supernatant, changed by less than 5% of the total fluorescence signal over a 10 min period. Continuous fluorescence signal (emission at 523 nm, excitation at 500 nm) was recorded in a Jobin-Yvon JY3 spectrofluorimeter equipped with a magnetic stirrer. The fluorescence signal was calibrated to yield pH_i by the following protocol. At the end of each experimental procedure, pH_i and the outside pH (pH_o) were equilibrated by permeabilizing the cells with 70 μM digitonin. Then the solution was titrated with either 0.1 M HCl or NaOH over the range of fluorescence values obtained during the experiment. By measuring the solution pH after each addition of acid, a calibration curve of fluorescence vs. pH was constructed for each experimental sample. Because the excitation peak of intracellular BCECF may exhibit a red shift, we constructed similar calibration curves by using an other permeabilizing agent, nigericin in the presence of high $[K^+]$. The correction factor thus obtained was about +0.05 pH units. Since the main purpose of using BCECF was to monitor changes in pH_i, the values reported here with digitonin calibration were not corrected to those obtained with nigericin [7].

Determination of buffering power

Two methods were used for determining the cytoplasmic buffering power.

(a) Direct measurement. The cell lysate was used for titration with NaOH, taking into account the buffering capacity of the medium. The intracellular and extracellular spaces were determined by isotopic method as described above.

(b) NH_4Cl titration. After equilibration of BCECF-loaded cells, 5–15 mM NH_4Cl was added and pH_i was recorded. The buffering capacity was calculated as $\Delta[NH_4^+]_i/\Delta pH_i$. The concentration of $[NH_4^+]_i$ was calculated using a pK_a of 9.21 and assuming that NH_3 is in equilibrium across the membrane. Penetration of NH_4^+ was corrected by back extrapolation [2,20].

Statistics

Values are presented as means ± S.E. Comparisons between group means were evaluated by the unpaired *t*-test.

Results

Cytoplasmic pH and buffering capacity

Fig. 1 shows pH variations after digitonin disruption of intestinal cells suspended in lightly buffered K^+ medium. The pH_o null-point for cells preincubated for 45 min at 37°C in Hanks' medium (pH 7.3) indicated a pH_i of 6.97 ± 0.07. Similar results were found with other batches of similarly treated cells. There was no measurable difference when the cells were permeabilized in lightly buffered Hanks' solution. Measurements of pH in lysates, carried out as soon as possible after breaking cells, furnished a mean value of 6.73 ± 0.12 ($n = 5$). The readings, however, were steadily falling, presumably owing to metabolic acid production, and therefore represent lower limits for pH_i.

The method based on the distribution of the weak acid [^{14}C]DMO in the intracellular and extracellular spaces was used as reference for measuring cytoplasmic pH. The mean value obtained with ten different preparations was 7.11 ± 0.05. The measurement of intracellular and extracellular water, 11.2 ± 0.7 and 13.6 ± 1.0 μl/mg protein, respectively, confirmed previously reported values [18]. Measurements of cytoplasmic pH carried out with the dye BCECF furnished a mean value of 7.03 ± 0.05 ($n = 42$) when intestinal cells were suspended in Hanks' solution (pH = 7.3 at 37°C). This value is not significantly different from that obtained with DMO ($P > 0.1$). The cytoplasmic pH thus measured was stable for at least 5 min, but there were some differences among animals (range 6.9 to 7.2).

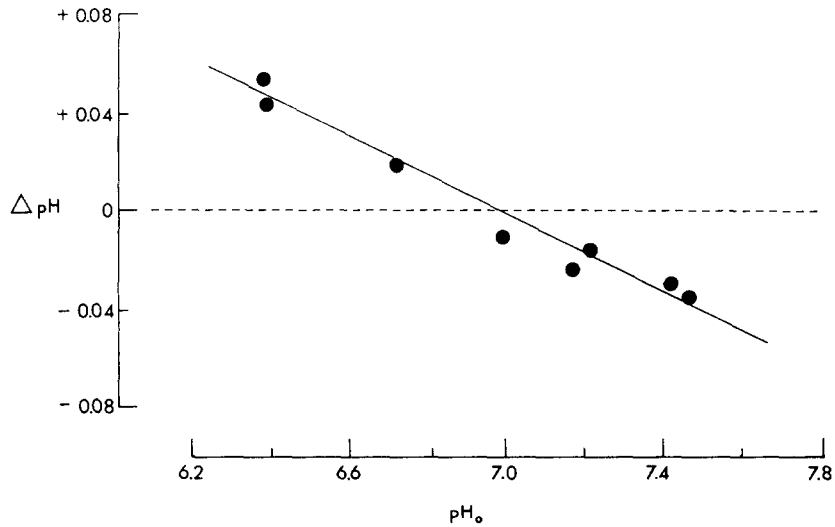

Fig. 1. pH_0 null-point for digitonin permeabilization of the membrane of rat enterocytes. Vertical axis shows the change in pH of lightly buffered medium on addition of 70 μM digitonin. Cells (1 mg cellular protein/ml) were preincubated in Hepes-buffered Hanks' medium at pH 7.30.

Different methods were used to determine the internal buffering power of intestinal cells. The first and simplest involved the direct titration of cells disrupted with a homogenizer. The buffering power thus determined was 36 ± 11 mmol $\cdot l^{-1}$.

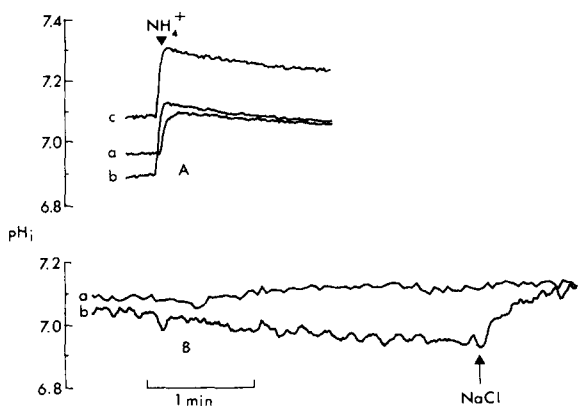

Fig. 2. (A) Determination of the buffering power of enterocytes by NH_4^+ titration. Cells were loaded with BCECF as described and suspended in Hanks' medium (pH 7.30) (1 mg cellular protein/ml). 5 mM (a), 10 mM (b) or 15 mM (c) NH_4Cl was added and pH_i was recorded immediately. (B) Effects of Na^+ and Na^+-free treatment on pH_i in control enterocytes. (a) Cells were resuspended in Hanks' medium, and the pH_i remained constant. (b) Cells were resuspended in choline Cl solution and the pH_i decreased. This acidification was reversed when 100 mM NaCl was added back (arrow) to the solution. All traces represent one of five identical experiments.

pH^{-1}. Fig. 2A illustrates the second method used to determine buffering power. The increase in pH_i following the addition of NH_4Cl was measured in cells at different pH_i values. As seen (Fig. 2A), rapid alkalinization was followed by a slow acidification, probably due to the influx of protonated base. The precise measurement of the alkalinization induced by NH_3 uptake was obtained by back-extrapolation of this secondary acidification to the time of addition of extracellular NH_4^+ [20]. The values obtained for different pH_i values at equilibrium and for different NH_4Cl concentrations (5–15 mM) were much higher than those found after direct titration: average value of 62 ± 5 mmol $\cdot l^{-1} \cdot pH^{-1}$ ($n = 7$).

Na^+/H^+ exchange

When BCECF-loaded intestinal cells were suspended in Hanks' medium, the fluorescence plot was stable for several minutes and the pH_i was close to 7.00. When cytosolic or medium molar sodium ion concentration was modified, however, cytoplasmic pH changed. Thus, when the medium lacked sodium (choline chloride medium), the pH_i decreased regularly with time (Fig. 2B). The acidification of cells was reversed when 100 mM NaCl was added to the medium. As seen in Fig. 3, the time-course of intracellular alkalinization is

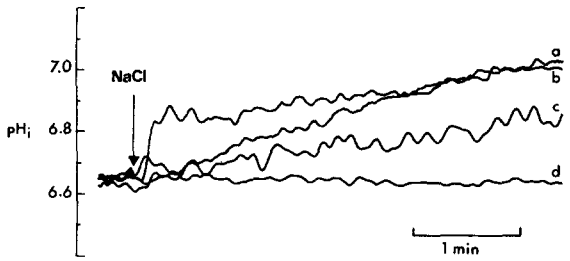

Fig. 3. Concentration-dependence of Na^+ effect on pH_i change. Isolated enterocytes were preincubated 15 min in choline chloride medium pH 7.30. NaCl was added to give an Na^+ final concentration of 100 mM (a), 50 mM (b), or 25 mM (c). As a control recording, 50 mM choline chloride was added (d).

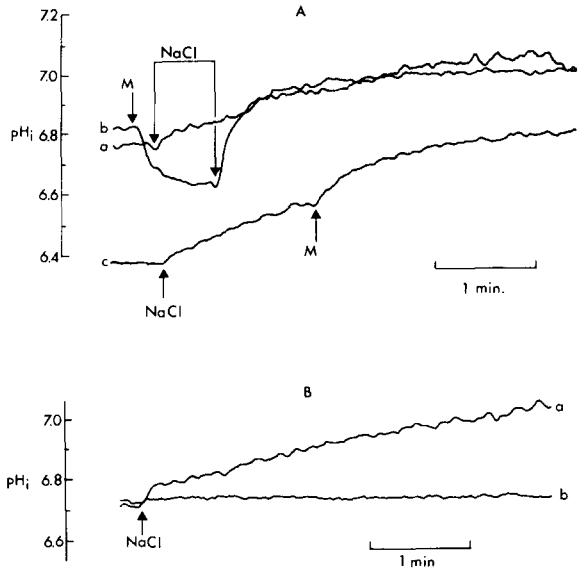

Fig. 5. (A) Effects of Na^+-free treatment in ouabain-treated or acidified cells. (a) Cells were incubated in Hanks' solution containing 0.3 mM ouabain for 45 min, then resuspended in choline chloride solution. Acidified cells did not regulate pH_i until 100 mM NaCl was added to the suspension. (b) When 10 μM monensin (M) was added to the ouabain-treated cells, the rate of acidification was more rapid. Adding 100 mM NaCl back to the solution reversed the monensin-induced acidification and the pH_i immediately increased back toward control pH_i levels. (c) When cells were treated with 30 mM NH_4Cl for 5 min and then washed quickly in choline chloride solution, enterocytes were initially rather acidic. Regulation of pH_i occurred only after addition of NaCl to the choline chloride solution. When 10 μM monensin (M) was added, the alkalinization became faster. (B) Effect of amiloride on the Na^+-induced change in pH_i. NaCl (50 mM) was added both in the absence (a), and the presence (b) of 1 mM amiloride. All traces represent one of five similar experiments.

dependent on the Na^+ concentration added. A high concentration of added Na^+ accelerated the alkalinization process. An identical result was obtained when NaCl was replaced by $NaCH_3SO_3$ (Fig. 4A). When cells were resuspended in Cl^- free solution ($NaCH_3SO_3$ medium) and when Cl^- was added, the pH_i remained constant (Fig. 4B).

When cells were incubated for 45 min in Hanks' medium containing 0.3 mM ouabain, the cytoplasmic pH was 7.04 ± 0.06. When they were resuspended in choline chloride medium, the cytoplasmic pH decreased by about 0.25 unit and when 100 mM NaCl was added, the pH_i returned to its initial value (Fig. 5A, trace a). A more rapid and more intense acidification was recorded after adding 10 μM monensin (an ionophore which

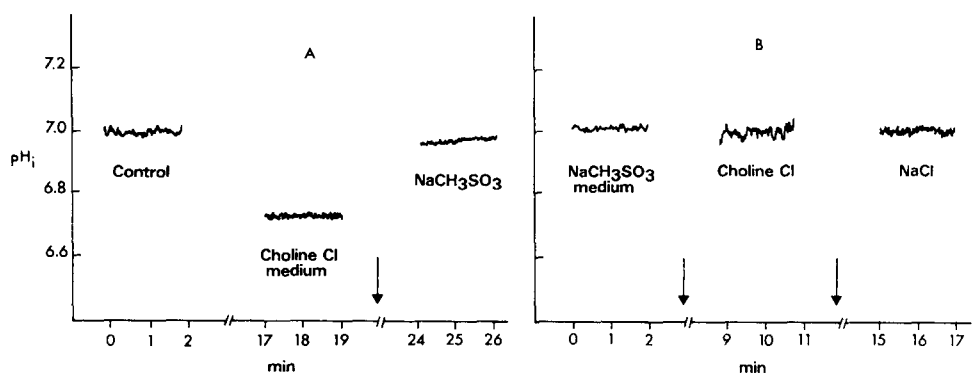

Fig. 4. Fluorescence trace of intestinal cells loaded with BCECF. (A) Isolated enterocytes were preincubated in Hanks' medium (pH 7.30) (control), and resuspended in choline chloride medium (pH 7.30) for 15 min. At the arrow, $NaCH_3SO_3$ (100 mM) was added and the pH_i returned to control value within a period of 5 min. (B) Isolated enterocytes were preincubated in $NaCH_3SO_3$ medium (pH 7.30) for 15 min. Arrows indicate the time of addition of choline chloride (100 mM), and NaCl (100 mM). The addition of Cl^- did not cause any significant change in pH_i.

artificially exchanges Na^+ for H^+). In this case as well, the addition of NaCl enabled the cells to recover their initial equilibrium pH (Fig. 5A, trace b). When the enterocytes were treated with 30 mM NH_4Cl for 5 min, and then rapidly resuspended in choline medium, the cells were initially rather acidic, but they rapidly regulated pH_i back to control levels after adding NaCl to choline chloride medium (Fig. 5A, trace c). The return of pH_i to its control level value was accelerated by monensin.

When acidified cells were treated with 0.1 mM amiloride, the Na^+-dependent regulation of pH_i was largely prevented (32% inhibition), but, as seen in Fig. 5B, 1 mM amiloride abolished the Na^+ induced changes in pH_i.

Discussion

Cytoplasmic pH and buffering power of intestinal cells

The present results show that cytoplasmic pH of isolated rat intestinal cells is close to 7.0. Among the four methods used, the only discordant results were obtained with the direct measurement of pH with an electrode, the pH_i being about 0.3 units more acid. The measured values were perturbed by the destruction of intracellular compartments (notably lysosomes) and above all by the continued production of acid by glycolysis or ATP hydrolysis. This simple method thus furnished only a crude estimation of the pH_i. The digitonin method, on the other hand, did not present the same disadvantages, since the compound does not destroy lysosomal or mitochondrial membranes [6]. The results obtained were reproducible and the mean value appeared to be a satisfactory estimation of cytoplasmic pH.

The method based on the distribution of the weak acid, DMO, is generally considered to be the reference method for evaluating cytoplasmic pH. It nevertheless has several limitations, especially the requirement for determining the volume of intracellular water. A slight error in determining this parameter could have a large effect on the calculated pH_i. In addition, the dissociation constant of DMO in intracellular water is not known and is taken as identical to that measured in extracellular water. The equation used shows that an error in estimation the pK_a induces an error of the same order in the calculated pH_i.

The fluorescent indicator, BCECF, trapped inside cells has several advantages for measuring pH_i in vitro. It is highly sensitive to slight changes in pH, relatively few cells are required for the determination, and it is compatible with different extracellular media tested. In addition, the dye is excluded by organelles [6,7] and so the measured pH is exclusively that of the cytoplasm. Finally, the extremely short resolution time of the method enables pH_i to be recorded continuously, as long as cell viability is unaffected by BCECF. The resting pH_i of intestinal cells was 7.03 ± 0.05 when the cells were suspended in physiological saline at pH 7.3. Available data on the pH_i of enterocytes [3,4] and other mammalian cells [2] are in good agreement with the results of the present work.

Of the two methods used to determine the buffering capacity of intestinal cells, that of directly titrating cell lysates is the more controversial. In addition to the drawbacks described above (primarily destruction of intracellular compartments), uncertainty is introduced because of the need to estimate the volume of extracellular contained in the sample. Titration with NH_4Cl is undoubtedly the best method for determining buffering capacity, since it utilizes the BCECF method with all its advantages, primarily the fact of using intact cells. Finally, experiments carried out for different initial pH_i values and NH_4Cl concentrations gave similar results and so the values obtained may be taken with confidence. Furthermore, the buffering capacity found, 62 ± 5 $mmol \cdot l^{-1} \cdot pH^{-1}$ is in the middle of the range of values measured for other tissues and listed by Roos and Boron [2].

Na^+/H^+ exchange

When an (out < in) Na^+ gradient was established, cells acidified (Fig. 2B, trace b), in contrast to cells in Hanks' medium containing 137 mM NaCl (Fig. 2B, trace a). This acidification was reversed when NaCl was added. The time-course of intracellular alkalinization is dependent of the external Na^+ concentration (Fig. 3). When Cl^- was replaced by $CH_3SO_3^-$ the pH_i was regulated in the same way (Fig. 4A). The absence of any

effect of Cl^- was confirmed when cells were incubated in Cl^--free medium (Fig. 4B).

Acidification of ouabain-treated cells was greater (0.25 pH unit) than that of normal cells (0.10 pH unit), probably because the Na^+ gradient was higher, the effect of ouabain (inhibitor of Na^+/K^+-ATPase) is to load the cells with sodium. After this acidification in a sodium-free medium (choline chloride), the addition of 100 mM NaCl enabled the pH_i to return to control values (normal cells: Fig. 2B, trace b; ouabain-treated cells: Fig. 5A, trace a). The phenomenon observed is the same as that obtained by adding 10 μM monensin, which is known to create an artificial Na^+/H^+ exchange [7]. In the latter case, however, the rate and amplitude of acidification, as well as the rate of return to control pH_i due to NaCl, are much greater (Fig. 5A, trace b). These observations, and the fact that 1 mM amiloride, an inhibitor of Na^+/H^+ exchange [10,21] completely blocks the return of pH_i to its normal value, indicates the presence of an Na^+/H^+ exchanger which operates reversibly as a function of the Na^+ gradient. The existence of this Na^+/H^+ exchanger was supported by the fact that we obtained practically the same results with cells acidified by the technique of NH_4^+ loading. Thus, the cells became acid when NH_4Cl was removed from the incubation medium by washing, but they regulated their pH_i in the presence of NaCl (Fig. 5A, trace c). Again, in this case, the addition of 10 μM monensin accelerated the return of the pH_i to control values.

It may thus be concluded that isolated intestinal cells contain an Na^+/H^+ exchanger which appears to be independent of Cl^- and which is inhibited by 1 mM amiloride. The presence of this type of exchanger has already been shown in different mammalian cell types [7,20,22]. This type of regulation in intestinal cells has been shown for the brush borders of rabbits [10] and rats [9] and for isolated enterocytes of rabbits [4]. Nevertheless, even though the present study confirmed the Na^+/H^+ exchange, it showed that regulation is slower and weaker than that of the brush border. In isolated intestinal cells, however, the presence of an Na^+/H^+-exchanger in the basolateral membranes [11], as well as other types of regulation, e.g., K^+/H^+ exchanger [23] and K^+ conductance channels [9], but also the presence of acidic microclimate pH layer in contact with the luminal surface of the enterocytes [24,25], could partially compensate the Na^+/H^+ exchanger effect. In addition, the cytoplasmic contents were intact in our experimental model, and so the existence of subcellular compartments containing Na^+/H^+ and K^+/H^+ exchangers [26], as well as the high value of cytoplasmic buffering power, could explain the low measured variations of pH_i. The present experimental model used intact viable cells and not isolated membranes and so enabled the pH_i and its regulation to be expressed as they must exist in intestinal cells in vivo.

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