Biochimica et Biophysica Acta 901 (1987) 265–272
Elsevier

BBA 73616

Role of Na^+/H^+ antiport in intracellular pH regulation by rabbit enterocytes

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(Received 5 December 1986)
(Revised manuscript received 10 April 1987)

Key words: pH, intracellular; Enterocyte; Antiport, Na^+/H^+; Fluorescence; (Rabbit)

The steady-state intracellular pH (pH_i) of isolated rabbit enterocytes was determined using 9-aminoacridine, a fluorescent weak base, and the null-point method with digitonin. When cells are incubated in a Na^+-containing solution, the estimated value of pH_i was in the range of 7.10–7.20, whereas it was 6.60–6.70 when cells were incubated in a Na^+-free solution, indicating an important role of external Na^+ in maintaining pH_i at a slightly alkaline level. Pulse injection of Na^+ into a Na^+-free cell suspension induced a slowly developing alkalinization of pH_i. The time course of the alkalinization was found to be dependent on the Na^+ concentration. Li^+ had the same effect as Na^+, while K^+ had a slight effect. Amiloride inhibited the effects of Na^+ dose-dependently. These results indicate that the Na^+/H^+ antiport plays an important role in maintaining the pH_i at a neutral or slightly alkaline level in the intact enterocytes.

Introduction

Recent studies have shown that the Na^+/H^+ antiport mechanism located in cell membranes is playing an important role in the regulation of intracellular pH (pH_i) in a variety of cell types (for review, see Ref. 1). The presence of the Na^+/H^+ antiporter in the intestinal brush-border membranes has been shown by several authors using isolated membrane vesicles [2–5]. In the case of the enterocytes, the brush-border membrane Na^+/H^+ antiporter has been suggested to play an important role not only in pH_i regulation but also in maintenance of the microclimate pH in the close vicinity of the cell surface which is very important for intestinal absorption, particuarly for the H^+-dependent transport of dipeptides and tripeptides [6]. However, there is no experimental evidence of the activity of the Na^+/H^+ antiporter in the intact epithelial cells of the mammalian small intestine, particularly in regard to pH_i regulation. The present study has been designed to demonstrate the role of the Na^+/H^+ antiporter in pH_i regulation in the intact enterocytes isolated from rabbit.

Materials and Methods

Isolation of the enterocytes

The epithelial cells of rabbit small intestine were isolated by the following procedures. The animals (2.0–2.5 kg body weight) were killed by an intravenous injection of urethane, and the small intestine extending from the terminal ileum to the just distal to the ligament of Treitz was excised. The isolated intestine was divided into four segments of equal length, and each was everted by use of a polyethylene tube. The everted segments were washed in an ice-cold isotonic NaCl solution

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containing 0.5 mM DL-dithiothreitol, which is known to reduce the mucous contents [7]. Each segment was ligated at both ends and transferred into a buffer solution containing Dispase. This incubation medium had the following composition (in mM); Tris-HCl, 140; KCl, 2; KH_2PO_4, 3; $CaCl_2$, 1.8; $MgCl_2$, 1.0; D-mannitol, 10; β-hydroxybutyrate, 1.0; DL-dithiothreitol, 0.5 (pH 7.30). Dispase was added to the medium at 1000 p.u./ml. The incubation with Dispase lasted 20 min at 37° C under mild shaking. The suspension of isolated cell clusters was filtered through a nylon mesh, then centrifuged at $150 \times g$ for 2 min. The pellet containing isolated cells was resuspended in the same incubation medium but containing 1 mg/ml collagenase, instead of Dispase, and incubated for 5 min at 37° C. After the second incubation, the cell suspension was filtered through a nylon mesh again and washed twice with enzyme-free solution. The solution used for the resuspension and washing had the following composition (in mM): Choline chloride, 120; KCl, 3; $CaCl_2$, 1.8; MgCl, 1.0; D-mannitol, 50; β-hydroxybutyrate, 1.0; and Hepes/Tris, 20 (pH 7.30). After the second treatment, the cells were found to be mostly single. Trypan blue exclusion test for these cells revealed that more than 85% of cells were maintaining cell integrity. The cell suspension was kept at 4° C before the start of experiments. All experiments were performed within 90 min after preparation. Within this period, cell integrity was well maintained as indicated by Trypan blue exclusion tests.

Fluorescence measurement

Changes in transmembrane pH difference (ΔpH) were monitored by continuous recording of the fluorescence of 9-aminoacridine added to the cell suspension. A Hitachi 650-10S fluorescence spectrophotometer, equipped with a thermo-regulated cuvette with a continuous stirring system, was used. The excitation and emission wavelength were set to 400 nm and 454 nm, respectively. Band width was 5 nm in each wavelength. The cuvette was filled with 2 ml test solution, and 9-aminoacridine was added to the medium to a concentration of 10 μM. The fluorescence of the cell free medium which contained 10 μM 9-aminoacridine was adjusted to 100 arbitrary units, and the gain was kept constant throughout a series of experiments. Under our experimental conditions, light scattering from the cells were negligible.

At the beginning of experiments, an aliquot of the cell suspension was centrifuged and the cells were preincubated in one of the 'preincubation media', as described below, for 15 min at 37° C. Two different solutions were used for the preincubation, one the Na^+-containing, the other Na^+-free solution. The Na^+-containing preincubation medium had the following composition (in mM): NaCl, 120; KCl, 3; $CaCl_2$, 1.8; $MgCl_2$, 1.0; D-mannitol, 50; β-hydroxybutyrate, 1.0 and Hepes/Tris, 20 (pH 7.30). The Na^+-free preincubation medium contained 120 mM choline chloride instead of 120 mM NaCl. After the preincubation, the cells were centrifuged, resuspended in a test medium and transferred into a cuvette. The cell concentration in the cuvette was $5 \cdot 10^6$ cells/ml in all experiments. The compositions of the test media are mentioned in the results.

Materials

Dispase was obtained from Godo Shusei (Tokyo). Collagenase and digitonin were from Wako (Tokyo). DL-Dithiothreitol and nigericin was from Sigma. 9-Aminoiacridine was from Aldrich Chemical. Amiloride was a kind gift of Merck, Sharp and Dohme. All other chemicals were of reagent grade.

Results

pH-dependent quenching of 9-aminoacridine fluorescence in enterocyte suspension

9-Aminoacridine is known to distribute across cell or vesicular membranes in accordance with a pH difference (ΔpH) across the membranes [8–10]. To test the usefulness of 9-aminoacridine in estimation of pH_i or monitoring pH_i changes, we first examined the quenching of 9-aminoacridine fluorescence induced by addition of the cells and the dependence of the degree of quenching on the external pH (pH_o). The cells, preincubated in the medium of pH 7.30 for 15 min, were resuspended in 2 ml of one of the test media of different pH values (pH 6.80, 7.30, 7.80 and 8.30) at $5 \cdot 10^6$ cells/ml and transferred to a cuvette, to which

9-aminoacridine was added to give a final concentration of 10 μM. The test media had the following composition (in mM): NaCl (or choline chloride), 120; $CaCl_2$, 1.8; $MgCl_2$, 1.0; D-mannitol, 50; β-hydroxybutyrate, 1.0 and buffered with 20 mM Hepes/Tris (pH was adjusted to the above values). The fluorescence intensity of cell free medium which contained 10 μM 9-aminoacridine was set to 100 arbitrary units. Recording of fluorescence intensity was started from the time of addition of the amine and continued for 3–5 min until the fluorescence level had been almost stabilized. The presence of the cells caused a marked quenching of fluorescence. Although there was a large nonspecific quenching, it was also seen that the final level of fluorescence intensity was dependent on pH_o, as shown in Fig. 1. In this particular experiment, recording was made in the absence of Na^+ in the external medium (the choline test medium), but similar results were obtained in the presence of Na^+. Since all the cells were equilibrated with the same preincubation medium, pH_i of the cells were expected to be the same initially. Accordingly, the observed pH_o-dependence of quenching on external pH observed indicates that 9-aminoacridine distributes in accordance with ΔpH across the cell membranes.

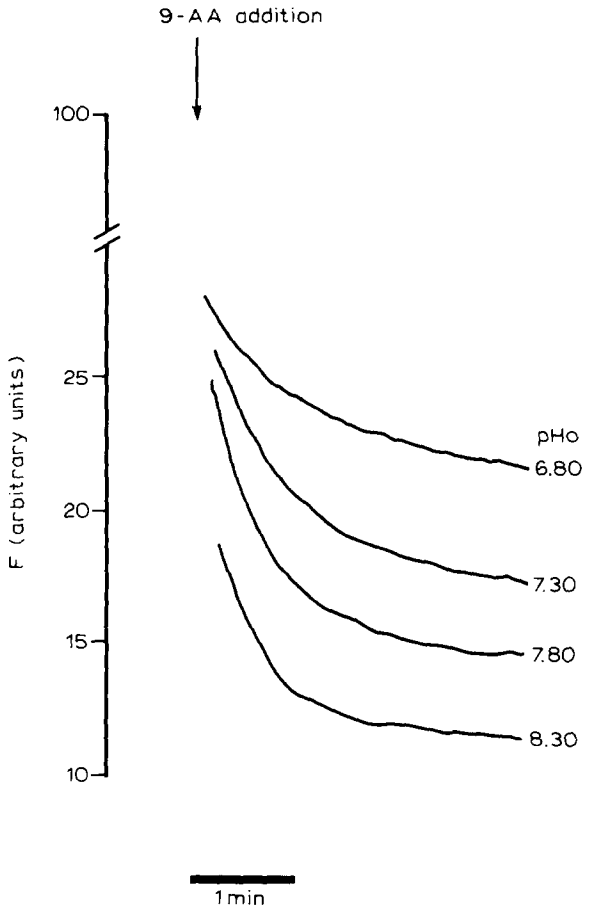

Fig. 1. Effect of pH_o on quenching of 9-aminoacridine (9-AA) (10 μM) induced by the presence of enterocytes at a constant concentration ($5 \cdot 10^6$ cells/ml). Isolated enterocytes were preincubated in choline medium of pH 7.30 and resuspended in choline test media of different pH values. The fluorescence intensity of cell-free blanks was set to 100 arbitrary units.

Measurements of pH_i by the null-point method

In the second series of experiments, we intended to determine pH_i of the enterocytes equilibrated with either the standard Na^+-containing or Na^+-free medium of pH 7.30. A null-point method was used for this purpose. Digitonin was added to the medium to increase plasma membrane permeability [11,12]. The isolated enterocytes were preincubated in the Na^+-containing or Na^+-free (choline-substituted) preincubation medium for 15 min prior to the application of the null-point method. The cells were washed and resuspended at $5 \cdot 10^6$ cells/ml in the medium containing 140 mM KCl, 10 μm 9-aminoacridine and 20 mM Mes/Hepes/Tris (pH 6.00–6.50) or Hepes/Tris (pH 6.5–8.3). 2 ml of the suspension was transferred into a cuvette, and the fluorescence was monitored for 2–3 min. Then, digitonin dissolved in DMSO was added to the suspension to give a final concentration of 75 μM. The final concentration of DMSO did not exceed 1% (v/v). The addition of the same dose of digitonin to a cell-free blank did not cause any significant change in the fluorescence. In contrast, digitonin caused an immediate change in fluorescence when cells were present (Fig. 2). The changes can be regarded as the results of rapid dissipation of a pH difference across the cell membrane. As the pH of the medium was unchanged throughout experiments because of a sufficiently strong buffering capacity of the medium (this was checked by a pH-meter in the separate experiments). pH_i was changed to equilibrate with pH_o after digitonin addition. No fluorescence change is expected to

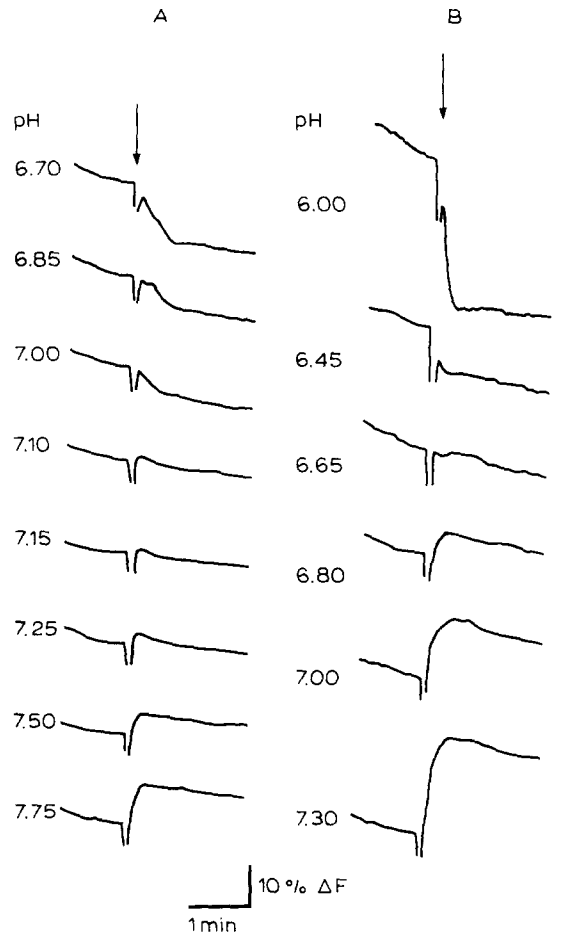

Fig. 2. Measurement of pH_i by the null-point method with digitonin. Isolated enterocytes were preincubated in Na^+ (A) or choline (B) medium of pH 7.30 and resuspended in KCl media of different pH values. The change in fluorescence upon the addition of digitonin (75 μM) was followed. Arrows indicate the time of addition of digitonin.

occur when pH_o is the same as pH_i. From finding such pH_o, we can estimate pH_i. Fig. 2 shows the effects of digitonin addition on the fluorescence of the enterocyte suspension at different values of pH_o. Tracings obtained from the cells preincubated in the Na^+-containing medium are shown on the left side (Fig. 2A) and those from the cells preincubated in the Na^+-free (choline-substituted) medium are on the other side (Fig. 2B). The null-points thus determined lie between 7.10 and 7.15 in the former and around 6.65 in the latter. Repeated experiments showed that the pH_i of cells preincubated in the Na^+-containing medium was 7.10–7.20 (experiments from four different animals), whereas the values of cells preincubated in the Na^+-free medium was 6.60–6.70 (experiments from three different animals). These results indicate that pH_i is dependent on the presence of Na^+ in the external medium.

Effects of monovalent cations on pH_i

The results described above indicate that external Na^+ is involved in extrusion of H^+ which maintains intracellular pH at a neutral or slightly alkaline level. To examine the role of Na^+ versus other cations, the effects on pH_i of addition of Na^+, Li^+ and K^+ were compared using the cells suspended in Na^+-free medium.

Before this series of experiments, we examined the change in the 9-aminoacridine fluorescence of the cell suspension in high KCl medium by addition of nigericin. Cells were preincubated in Na^+-containing preincubation medium of pH 7.30 and resuspended in the medium containing 140 mM KCl, 10 μM 9-aminoacridine and 20 mM Hepes/Tris (pH 7.80). Addition of Nigericin, which catalyzes K^+–H^+ exchange, is expected to cause an intracellular alkalinization under these experimental conditions. Fig. 3 shows that change in the 9-aminoacridine fluorescence was well in accordance with this expectation. Addition of digitonin shows that pH gradient across cell mem-

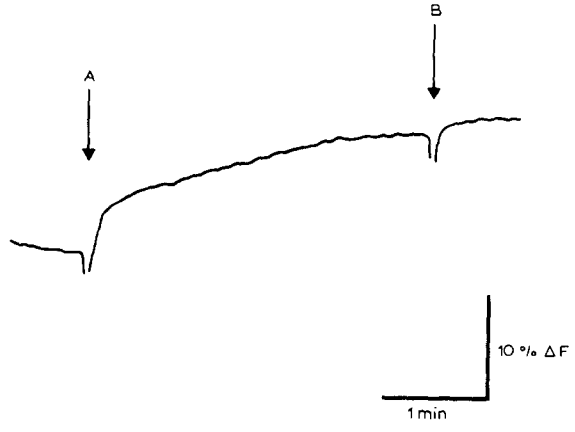

Fig. 3. Effect of addition of nigericin on the 9-aminoacridine fluorescence of the cell suspension. Isolated enterocytes were preincubated in Na^+ medium of pH 7.30 and resuspended in KCl medium of pH 7.80. At arrow A, nigericin (10 μM) was added and at arrow B, digitonin (75 μM) was added.

brane was almost dissipated by nigericin within this period.

The isolated cells were preincubated in the Na^+-free medium of pH 7.30. Fluorescence measurements were performed by resuspending cells in Na^+-free test medium of pH 7.80 containing 10 μM 9-aminoacridine. The composition of the Na^+-free test medium was as follows (in mM). Choline chloride, 120; $CaCl_2$, 1.8; $MgCl_2$, 1.0; D-mannitol, 50; β-hydroxybutyrate, 1.0 and Hepes/Tris, 20 (pH 7.80). After the stabilization of the fluorescent level, 20 μl of 2 M stock solution of NaCl, LiCl, KCl or choline chloride was added to the cuvette to give a final concentration of 20 mM. Choline addition was performed as a control. The injection of the concentrated 'cation' solution itself had a negligible effect on the fluorescence when tested in a cell-free medium. Also, pH of the medium was not changed by cation additions (checked by a pH-meter in the preliminary experiments).

Fig. 4 shows the time courses of changes in fluorescence of cell suspensions caused by addition of different monovalent cations. In this figure, four tracings obtained with Na^+, Li^+, K^+ and choline were superimposed. Digitonin (75 μM) was added at the end of each experiment to see the fluorescence level in the absence of a gradient. As seen in this figure, Na^+ and Li^+ caused slow

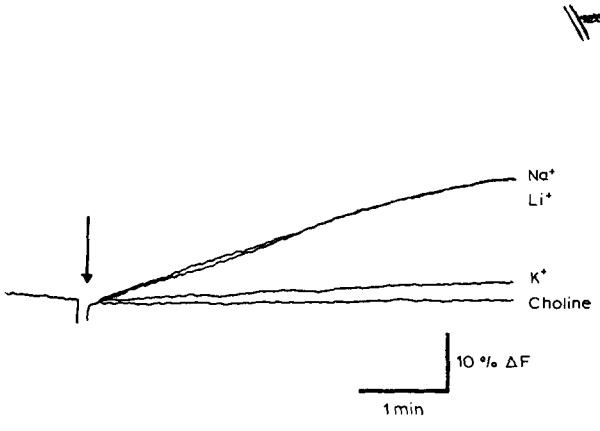

Fig. 4. Effects of addition of monovalent cations on 9-aminoacridine fluorescence. Isolated enterocytes were preincubated in choline medium of pH 7.30 and resuspended in choline test medium of pH 7.80. Each cation was added to give a final concentration of 20 mM. Digitonin (75 μM) was added at the end of each recording.

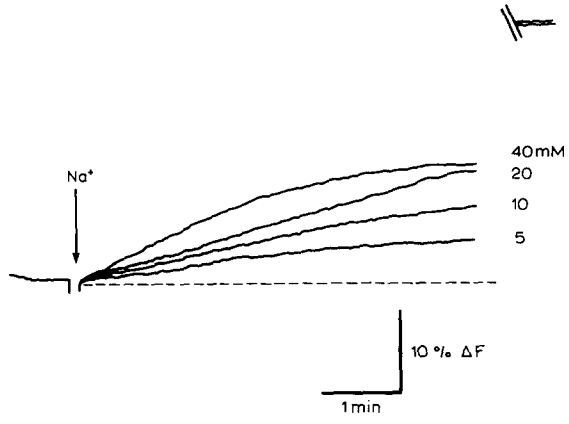

Fig. 5. Concentration-dependence of Na^+ effect on fluorescence change. Isolated enterocytes were preincubated in choline medium of pH 7.30 and resuspended in choline test medium of pH 7.80. As a control recording, 20 mM choline chloride was added. Digitonin (75 μM) was added at the end of an experiment.

increases in fluorescence (13% after 3 min). On the other hand, K^+ caused a smaller increase in fluorescence (2.4% after 3 min). Since choline had no effect, the effects of Na^+ and Li^+ are not due to the change in osmolarity or ionic strength of the medium. Therefore, the data indicate that external Na^+ and Li^+ stimulate H^+ extrusion.

The effect of the external Na^+ concentration on the time course of the intracellular alkalinization process is shown in Fig. 5. NaCl was added at different concentrations; 5, 10, 20 and 40 mM, and four tracings were superimposed. As seen in this figure, the time course of intracellular alkalinization is dependent on the Na^+ concentration added. A high concentration of added Na^+ accelerated the alkalinization process. A similar concentration-dependent time course of the intracellular alkalinization was also observed for Li^+ (data not shown).

Effect of amiloride on monovalent cation-induced intracellular alkalinization

Amiloride is known to be a potent inhiibtor of the Na^+/H^+ antiporter [1]. Fig. 6 shows the effects of 20 mM Na^+, Li^+, K^+ or choline on the fluorescence in the absence (control) and the presence of 10^{-4} M amiloride. The same Na^+-free test medium as that used in the experiments on the 'effects of monovalent cations' experiments was

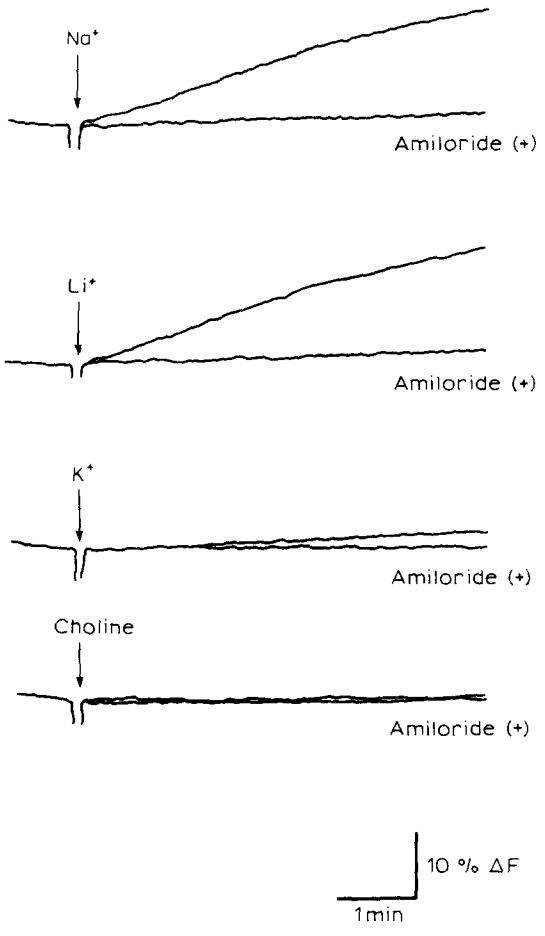

Fig. 6. Effect of amiloride on the cation-induced changes in fluorescence. The experimental procedure was the same as that of Fig. 4. The test cations were added both in the absence and the presence of 10^{-4} M amiloride.

used in this series of experiments. Two tracings obtained in the presence and absence of amiloride were superimposed in this figure. As seen in this figure, amiloride abolished the Na^+- and Li^+-induced fluorescence changes, indicating that the effects of Na^+ and Li^+ on H^+ extrusion are mediated by an amiloride-sensitive process, most probably by the Na^+/H^+ antiport mechanism. Amiloride also abolished the K^+-induced small changes in fluorescence, suggesting that K^+ is also able to interact with the Na^+/H^+ antiporter. Fig. 7 presents the dose-inhibition relationship observed for 20 mM Na^+-induced fluorescence changes. A detectable effect of amiloride appeared from 10^{-7} M, the maximum effect (almost 100% inhibition) occurred at 10^{-4} M, and the half maximum inhibition (ID_{50}) was at $3 \cdot 10^{-6}$ M.

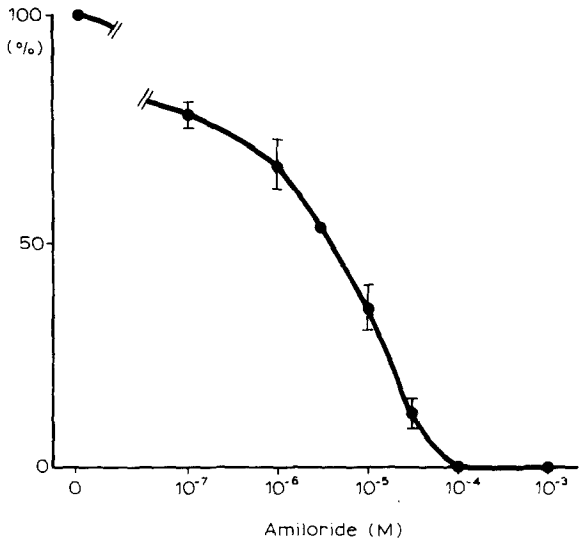

Fig. 7. Dose-response curve for amiloride inhibition of 20 mM Na^+-induced changes in fluorescence ($\Delta F/3$ min). The experimental procedure was the same as that of Fig. 6 except for the amiloride concentration. Values are shown as means ± S.E. from 3–5 experiments.

Discussion

The results of the present study show that the pH_i of rabbit enterocytes is dependent on external Na^+ and that the addition of external Na^+ stimulates H^+ extrusion out of the cells. With regards to the mechanism of the Na^+-dependent H^+ extrusion, amiloride-inhibition experiments, as well as the experiments on the specificity of the role of Na^+, indicate the importance of the Na^+/H^+ antiport. The presence of the Na^+/H^+ antiporters in intestinal brush-border membranes has been demonstrated by several authors using isolated membrane vesicle preparations [2–5]. The present study did not discriminate the roles of the brush-border and basolateral membranes since isolated single cells were used in our experiments. However, our results can support the suggestion that the Na^+/H^+ antiporters participate, at least in part, in maintenance of neutral or slightly alkaline pH_i in intact enterocytes. Moreover, this mechanism would contribute to the formation of the acidic microclimate pH layer in contact with

the luminal surface of the epithelial cells which was first documented by Lucas et al. [13].

The estimated value of pH_i of isolated rabbit enterocytes equilibrated with the Na^+-containing solution was found to be 7.10–7.20. The only available data of pH_i of enterocytes of other mammals are those obtained by Kurtin and Charney [14], who investigated in rat small intestine using the DMO method. They showed that pH_i was 6.83 in jejunal cells and 6.90 in ileal cells. For other epithelial cells, such as renal proximal tubules [15], pancreatic acinar cells [16], and colon epithelial cells [17], reported values of pH_i are ranging from 6.9 to 7.2. The value obtained in the present study falls in this range. Direct measurements of pH_i using a pH-sensitive microelectrode in newt small intestine revealed that pH_i was also slightly higher than 7.0 (7.2 on the average, Shimada and Hoshi, unpublished data). These observations indicate that the normal value of pH_i in enterocytes is around 7.0 or slightly higher than 7.0 although there may be slight variations depending on experimental conditions and regional differences. As the pH of the close vicinity of cell surface of rat small intestine is reported to be around 6.0 [13], a pH gradient of almost one pH unit is normally maintained across the brush-border membrane. Such a pH gradient together with cell-inside negative transmembrane potential has a particular importance in interpretation of highly preferential absorption of small peptides [18] since the transport of di- and tripeptides across the brush-border membrane is coupled with H^+ and driven by H^+ gradient across the membrane [19,20].

In the present study, we used 9-aminoacridine as a fluorescent probe for pH_i change. This amine is known to distribute in intra- and extracellular spaces according to the pH difference across the cell membrane. One problem in the use of such a weak base is the inhomogeneous distribution in the intracellular space, that is, the amine may be concentrated in more acidic compartments [21]. In a preliminary experiment, some of the isolated enterocytes equilibrated with 9-aminoacridine had several high fluorescent spots in the cytosol in the fluorescent microscope. Therefore, it should be said that the estimated values of pH_i by digitonin null-point method may represent the mean values of pH of the cell interior. For this reason, the use of another fluorescent probe for pH_i measurement, 2,7-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF) [12], was also tested in a preliminary experiment. We applied the acetoxymethylester of BCECF to the enterocyte suspension, but unfortunately, failed to load sufficient BCECF into the enterocyte interior. Rink et al. [12], who compared both a digitonin null-point method and BCECF method in lymphocytes, showed that there was no significant difference in estimated value of pH_i between these two methods.

In the present study, the role of other ions, such as HCO_3^- and Cl^-, in the pH_i regulation was not investigated and attention was focused on the role of the Na^+/H^+ antiport. The pH_i may be regulated by complex mechanisms involving Cl^-/HCO_3^- or Cl^-/OH^- exchange mechanisms. To delineate the whole mechanism, further studies are needed. However, the present study showed that the Na^+/H^+ antiport is functioning as a pH_i regulating mechanism in intact mammalian enterocytes.

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