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Heterogeneity of the Na^+-H^+ antiport systems in renal cells

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This study analyzes the differential characteristics of the Na^+-H^+ antiport systems observed in several epithelial and non-epithelial renal cell lines. Confluent monolayers of LLC-PK_{1A} cells have a Na^+-H^+ antiport system located in the apical membrane of the cell. This system, however, is not expressed during cell proliferation or after incubation in the presence of different mitogenic agents. In contrast, confluent monolayers of $MDCK_4$ express minimal Na^+-H^+ antiport activity in the confluent monolayer state but reach maximal antiport activity during cell proliferation or after activation of the cells by different mitogenic agents. Similar results were obtained with the renal fibroblastic cell line BHK. The system present in $MDCK_4$ cells is localized in the basolateral membrane of the epithelial cell. In LLC-PK_{1A} cells, an increase in the extracellular Na^+ concentration produces a hyperbolic increase in the activity of the Na^+-H^+ antiporter. In $MDCK_4$ and BHK cells, however, an increase in external Na^+ produces a sigmoid activation of the system. Maximal activation of the system occur at a pH_o 7.5 in LLC-PK_{1A} cells and pH_o 7.0 in $MDCK_4$ cells. The Na^+-H^+ antiporter of LLC-PK_{1A} cells is more sensitive to the inhibitory effect of amiloride (K_i $1.8 \cdot 10^{-7}$ M) than is the antiporter of $MDCK_4$ cells (K_i $7.0 \cdot 10^{-6}$ M). Moreover, 5-(*N*-methyl-*N*-isobutyl)amiloride is the most effective inhibitor of Na^+-H^+ exchange in LLC-PK_{1A} cells, but the least effective inhibitor in $MDCK_4$ cells. Conversely, the analog, 5-(*N*,*N*-dimethyl)amiloride, is the most effective inhibitor of Na^+-H^+ exchange in $MDCK_4$ cells, but is the least effective inhibitor in LLC-PK_{1A} cells. These results support the hypothesis that Na^+-H^+ exchange observed in LLC-PK_{1A} and other cell lines may represent the activity of different Na^+-H^+ antiporters.

Introduction

The existence of a Na^+-H^+ exchange system was originally proposed several years ago as a mechanism to explain the process of urine acidification [33]. The notion that urine acidification might occur by Na^+-H^+ exchange was later directly supported by demonstration of a Na^+-H^+ antiporter in renal microvillus membrane vesicles that mediated the uphill efflux of H^+ coupled to the downhill influx of Na^+ [32]. This system is not found in the basolateral plasma membrane of renal proximal tubular cells [25], indicating that during transepithelial transport, hydrogen ions move across each of these domains of the plasma cell membrane through two totally different transport systems. More recent studies have demonstrated that the Na^+-H^+ antiporter is not unique to epithelial membranes and the system can be found not only in other eukaryotic [3,9,16], but also in prokaryotic cellular systems [22,51]. In addition to urine acidification, the occurrence of Na^+-H^+ exchange has been implicated in a variety of cellular functions, including regulation of cytoplasmic pH [17], platelet activation [15,46], cell volume regulation [9,14], mitogenic activation [30,35], meiotic maturation of oocytes [12], metabolic response to hormones [31], and neutrophil activation [18]. It is clear from these studies that the Na^+-H^+ exchange system is widely, if not universally, distributed and involved in multiple and sometimes unrelated cellular functions. Despite the intensive characterization of the system in the last few years, it is unclear whether these multiple cellular functions result from the operation of a single system or if they represent the activity of different Na^+-H^+ antiporters. It is possible, even though they share the common feature of inhibition of Na^+-H^+ exchange by amiloride, that each one of the cellular functions associated with this exchange may actually represents the operation of a different antiport system. On the contrary, they may also represent the

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operation of a single antiport system but controlled by different regulatory mechanisms. To establish the similarities and differences between these systems, we analyzed the changes in activity of the Na^+-H^+ antiport induced by cell proliferation, different mitogenic agents, extracellular hydrogen and sodium ions concentration and amiloride and several of its analogs in several epithelial and nonepithelial cell lines. The results support the hypothesis that Na^+-H^+ exchange observed in LLC-PK_{1A} and other cell lines may represent the activity of different Na^+-H^+ antiporters. Preliminary reports of this study have been published [40,49].

Materials and Methods

Cell culture and monolayer preparation

LLC-PK_1 cells in the 198th passage and BHK cells in the 56th passage were obtained from the American Type Culture Collection and maintained by serial passages in 10-cm-diameter plastic tissue culture dishes (Costar, Cambridge, MA). A clone isolated from MDCK cells obtained from the American Type Culture Collection and designated clone 4 was kindly provided by Dr. J. Lever (University of Texas Medical School, Houston, TX). The cells were fed with Dulbecco's modified Eagle's medium containing 2 mM glutamine and 25 mM glucose. In addition, the medium was supplemented with 10% fetal bovine serum. All cultures were maintained in an atmosphere of 36 mmHg pCO_2 and 143 mmHg pO_2 at 37°C. When the cultures reached saturation density, subcultures were prepared using a 0.02% EDTA, 0.05% trypsin solution. Confluence was defined by the number of cells per cm^2 of monolayer. Two clones designated LLC-PK_{1A} and LLC-PK_{1B4} were isolated in the 225th passage from the parent LLC-PK_1 cell line as described previously [28]. These clones were selected based on the extent of Na^+-dependent sugar transport and Na^+-H^+ exchange exhibited in comparison with the parent cell line. Clone LLC-PK_{1A} used in the present study at confluence had a Na^+-H^+ exchange rate 250% higher than the parent cell line, while in the clone LLC-PK_{1B4} the exchange rate was only 20% of that observed in the parent cell line. In addition LLC-PK_{1B4} cells express undetectable Na^+-sugar cotransport and alkaline phosphatase and γ-glutamyl transpeptidase activities [51]. Although the number of passages of cells used in this study was reduced to a minimun of twenty, some variability in the Na^+ uptake was still evident. Monolayers grown on a permeable support were prepared using polycarbonate filter membranes (5 μm pore size and 25 mm diameter. Nuclepore Corp., Pleasanton, CA). The filter membranes were covered with a very thin film of a 0.5% collagen dispersion (Ethicon, Sommerville, NJ) and then applied to the bottom of the wells of a six-well tray (Costar, Cambridge, MA). The collagen was precipitated and aggregated into mature bundles with ammonia fumes. This procedure resulted in adhesion of the filters to the dish. After drying at room temperature, the collagen-coated membranes were gas sterilized with ethylene oxide. The total thickness of the permeable support is only 12 μm and has negligible effects on the diffusion of different solutes [39].

Electrolyte solutions

The uptake experiments were performed in a modified Earle's balanced salt solution (EBSS). The composition of this solution was similar to the electrolyte composition of the culture medium (millimolar): Na^+, 143; K^+, 5.36; Mg^{2+}, 0.8; Ca^{2+}, 1.8; Cl^-, 135; SO_4^{2-}, 1.0; Hepes, 10. To avoid Na^+ movement through the Na^+-phosphate or bicarbonate cotransport systems [36], the PO_4^{3-} and HCO_3^- buffers from the original EBSS solution were replaced by 10 mM Hepes. The pH was adjusted to 7.5 or 6.0 with 1 M NaOH or 1 M HCl.

Preincubation conditions

The uptake experiments were performed in monolayers incubated at 37°C. The monolayers were preincubated for 10 min in the presence of 10^{-3} M ouabain and then transferred to an EBSS solution in which the Na^+ was completely replaced by K^+ (K-EBSS). The pH of this solution was either 7.5 or 6.0 and contained an additional 4 μg/ml of nigericin as the H^+ ionophore. After a 5-min incubation, the ionophore was quenched with a 30-s wash in K-EBSS without nigericin but containing 0.5% bovine serum albumin at pH 7.5 or 6.0. At the end of this preincubation period the intracellular pH had reached equilibrium with the incubation solution [10,51], and the intracellular Na^+ concentration had diminished to undetectable levels. No differences in the activity of the Na^+-H^+ antiporter were observed after equilibration of the intracellular pH in presence or absence of nigericin [51]. This observation indicated that preincubation with the ionophore had not direct effect on the actual influx measurements.

Influx measurements

The ^{22}Na uptake assays were performed at 37°C by transferring the monolayers from the preincubation solution to the uptake solution at time zero. Only one group of experiments was performed at an extracellular Na^+ concentration of 143 mM (see Table IV). To reduced the contribution of the amiloride insensitive Na^+ influx, however, all other experiments were performed at a Na^+ concentration of 1.43 mM. The uptake solution was a modified EBSS solution in which NaCl was partially replaced by KCl to obtain a Na^+ concentration of 1.43 mM. The uptake solution also contained ^{22}Na as NaCl (1 μCi/ml). At the end of the

uptake period (2 min), the filters containing the cell monolayers were washed for 90 sec in ice-cold 0.1 M $MgCl_2$ solution. No significant label loss was observed from the intracellular compartment after this washing procedure [10]. After solubilisation with tissue solubilizer (NCS tissue solubiliser, Amersham Corp.), the radioactivity of the samples was measured in a Beckman liquid scintillation spectrometer Model LS-230 (Beckman Instruments). Corrections for interstitial trapping measured by the amount of [^{14}C]inulin associated with each sample in parallel experiments were minimal and not considered in the calculation of Na^+ influx. The sodium influx mediate by Na^+-H^+ antiporter was measured as the difference between the $^{22}Na^+$ influx measured in the presence (pH_i 6.0, pH_o 7.5 for LLC-PK_{1A} cells and pH_i 6.0, pH_o 7.0 for $MDCK_4$ cells) or absence of an outwardly oriented H ion gradient (pH_i 7.5, pH_o 7.5). The polarized uptake of ^{22}Na from the apical or basolateral side was determined on monolayers of $MDCK_4$ cells mounted between two hemichambers as described previously [10,37,51]. The solutions bathing both surfaces of the monolayers were of identical composition, with the exception of ^{22}Na, which was added selectively to either the apical or basolateral solution. After a 2-min uptake period at 37°C, both solutions were removed and the membrane was washed in ice-cold 0.1 M $MgCl_2$ solution. The radioactivity of the samples was determined by liquid scintillation counting as described above. The integrity of the monolayer during the uptake period was controlled by measuring the transepithelial electrical resistance as described below. No radioactivity was detected in the contralateral side after the 2-min uptake. The results were normalized for DNA content.

Electrical measurements

The monolayers grown on collagen-coated filters were removed from the culture dish and placed between two Lucite half-chambers with a window area of 3.14 cm^2. To reduce edge damage, the filters were placed between two silicone rubber rings coated with high vacuum grease (Dow Corning, Midland, MI). Each chamber contained 8 ml solution which was stirred gently with a magnetic bar. Transepithelial potential difference was measured with a high impedance Keithley Model 616 digital electrometer (Keithley Instruments, Cleveland, OH). We used pairs of Ag/AgCl electrodes placed 2 mm from the edge of the tissue. The total asymmetry with EBSS bathing both sides of the filters in the absence of monolayers was never more than 0.2 mV and was balanced out in the recording equipment. The current intensity in the conductance determinations was measured with a Weston microammeter Model 622 (Weston Electrical Instruments, Newark, NJ) and conducted by Ag/AgCl electrodes placed at opposite sides of the membrane at the distal ends of the chamber. A constant current was delivered with an operational amplifier (Philbrick Researchers, Boston, MA) set to deliver 500 μA. All voltage measurements were corrected by the voltage drop in the saline solution between the Ag/AgCl electrodes used to monitor the potential difference.

DNA assays

The DNA content was measured by a fluorometric micromethod described previously [38]. The rate of DNA synthesis was determined by measuring the rate of incorporation of [^{3}H]thymidine into the trichloroacetic acid precipitable material as described previously [28].

Reagents and supplies

Culture medium, fetal bovine serum, and trypsin-EDTA solution were purchased from GIBCO Laboratories, Grand Island, NY. ^{22}Na as NaCl and [^{3}H] thymidine were obtained from New England Nuclear, Billerica, MA. All plastic glasswares were from Costar, Cambridge, MA. All other reagents were analytical grade and were purchased from Sigma Chemical Co., St. Louis, MO.

Amiloride analogs

5-(*N*-Ethyl-*N*-isopropyl)amiloride (I), 5-(*N*,*N*-dimethyl)amiloride (II), 5-(*N*-methyl-*N*-isobutyl)amiloride (III), 5-(*N*-*tert*-butyl)amiloride (IV) and 5-(*N*-methyl-*N*-isobutyl)amiloride (V) were synthesized for this study using the previously described method [13].

Statistical analysis

Data were expressed as means ± S.E. Differences between controls and experimental values were analyzed with the paired Student's *t*-test. A value of $P < 0.05$ was considered significant. *P* values were given only for significantly different data.

Results

Changes in the Na^+-H^+-antiport activity during proliferation of LLC-PK_{1A} and $MDCK_4$ cells

Fig. 1 shows the changes in the Na^+-H^+-antiport activity of cultures plated with the number of cells necessary to attain 10% of the cell density observed at saturation density. The Na^+-H^+-antiport activity (measured as the difference between the $^{22}Na^+$ influx determined in the presence (pH_i 6.0, pH_o 7.5 for LLC-PK_{1A} cells and pH_i 6.0, pH_o 7.0 for $MDCK_4$ cells) or absence of an outwardly oriented H ion gradient (pH_i 7.5, pH_o 7.5) of these two cell lines is quite different. In $MDCK_4$ cells, it increases from values close to zero at the time of plating (zero time) to a maximal value above 35 μmol h^{-1} mg DNA^{-1}, three days after plat-

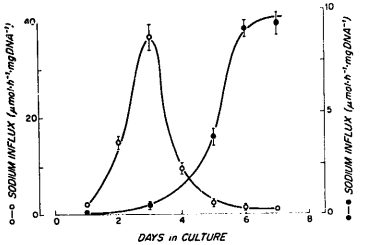


Fig. 1. Sodium influx through the Na^+-H^+ antiporter was measured as the difference between the $^{22}Na^+$ influx determined in the presence (pH_i 6.0, pH_o 7.5) or absence of an outwardly oriented H^+ gradient (pH_i 7.5, pH_o 7.5). The sodium influx was measured at different times after plating in $MDCK_4$ (open circles) and LLC-PK_{1A} cells (closed circles). Cells were plated at zero time with the cell number necessary to obtain a cell density (cells per cm^2) equal to 10% the saturation density (cells per cm^2 at confluence).

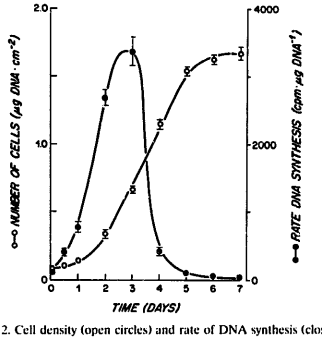


Fig. 2. Cell density (open circles) and rate of DNA synthesis (closed circles) measured at different times after plating $MDCK_4$ cells at 10% saturation density.

ing. From the third day of culture on, however, the activity of the antiporter starts to decrease, reaching very small values from the fifth day of culture, which are sustained to the end of the experimental period. In contrast, the activity of the Na^+-H^+ antiporter in LLC-PK_{1A} cells is minimal from the time of plating up to the fourth day of culture. From then on, the activity of the system starts to increase, reaching a maximal and steady-state value after the sixth day of culture.

Changes in cell density and rate of DNA synthesis during proliferation of $MDCK_4$ cells

Fig. 2 shows the changes in the rate of DNA synthesis and cell density in cultures of $MDCK_4$ cells plated at zero time and at 10% saturation density. The rate of DNA synthesis in these sparsely plated cultures increases, reaching a maximum 2 to 3 days after plating. Then, as the cultures reach saturation density, the rate decreases to values close to zero 4 days after plating. Considered together, the results presented in Figs. 1 and 2 indicate that the Na^+-H^+ antiport system in $MDCK_4$ cells is expressed only when the cells are in active proliferation: Confluent and mature monolayers do not express this antiport system.

Changes in cell density and rate of DNA synthesis during proliferation of LLC-PK_{1A} cells

Fig. 3 shows the changes in the rate of DNA synthesis and cell density in cultures of LLC-PK_{1A} cells plated at 10% saturation density. Like $MDCK_4$ cells, the rate of DNA synthesis increases from the time of plating (time zero) to a maximal value 2 to 3 days after plating. From then on, the rate decreases to values close to zero after the fifth day of culture. The cell density reflects the changes in the rate of DNA synthesis and reaches a maximal and steady-state value 5 days after plating. Comparison of the results presented in Figs. 1 and 3 indicates that LLC-PK_{1A} cells in active proliferation do not express the Na^+-H^+ antiport system. In clear contrast with the results obtained in $MDCK_4$ cells, the Na^+-H^+ antiport system is expressed only in confluent and mitotically arrested LLC-PK_{1A} cells.

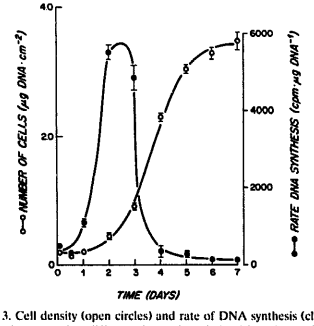


Fig. 3. Cell density (open circles) and rate of DNA synthesis (closed circles) measured at different times after plating LLC-PK_{1A} cells at 10% saturation density.

TABLE I

Changes in the Na^+-H^+ antiport activity during proliferation of LLC-PK_{1B4} and BHK cells

Sodium influx through the Na^+-H^+ antiporter was measured as the difference between the $^{22}Na^+$ influx determined in the presence (pH_i 6.0, pH_o 7.5) or absence of an outwardly oriented H^+ gradient (pH_i 7.5, pH_o 7.5). The results are the means ± S.E. of six monolayers.

Culture time (days)	Sodium influx (μmol h^{-1} mg DNA^{-1})	
	LLC-PK_{1B4}	BHK
1	2.53 ± 0.58	3.85 ± 0.40
2	16.14 ± 0.97	9.41 ± 0.77
3	34.22 ± 1.71	10.88 ± 1.48
4	9.41 ± 0.37	2.87 ± 0.73
5	1.29 ± 0.13	1.07 ± 0.03

Changes in the Na^+-H^+ antiport activity during proliferation of LLC-PK_{1B4} and BHK cells

Table I shows the changes in the Na^+-H^+ antiport activity of cells plated at 10% saturation density. The activity of the antiport system was measured as described in Methods as the difference between the $^{22}Na^+$ influx measured in the presence (pH_i 6.0, pH_o 7.0] or absence of an outwardly oriented H^+ gradient (pH_i 7.0, pH_o 7.0). Like $MDCK_4$ cells, the activity of the Na^+-H^+ antiport system in both LLC-PK_{1B4} and BHK cells attains maximal expression when the cells are in active proliferation three days after plating. Like $MDCK_4$1 confluent monolayers of LLC-PK_{1B4} and BHK cells express minimal, if any, Na^+-H^+ antiport activity.

Effect of serum on activation of the Na^+-H^+ antiport system of LLC-PK_{1A}, $MDCK_4$ and BHK cells

Table II shows the effect of incubation in the presence of 10% fetal bovine serum on the activity of Na^+-H^+ exchange in LLC-PK_{1A}, $MDCK_4$, and BHK cells. Prior to incubation in the presence of serum, the cells were maintained for 4 h in serum-free Dulbecco's medium. The activity of the antiport system was measured as described above 60 min after returning the cells to the serum-containing medium. The results show that incubation in the presence of 10% fetal bovine serum activates the Na^+-H^+ antiport system in BHK and $MDCK_4$ cells, but not in LLC-PK_{1A} cells.

Effect of phorbol 12-myristate 13-acetate on the activity of the Na^+-H^+ antiport system of LLC-PK_{1A}, $MDCK_4$ and BHK cells

Table III shows the effect of phorbol 12-myristate 13-acetate (TPA) on the activity of the Na^+-H^+ antiport system observed in LLC-PK_{1A}, $MDCK_4$ and BHK cells. The results show that incubation in the presence of TPA at a concentration of 10^{-6} M for 15 min produces significant stimulation of Na^+-H^+ exchange in proliferating $MDCK_4$ and BHK cells but not in LLC-PK_{1A} cells. The effect of TPA on $MDCK_4$ and BHK cells is associated with an increase in the rate of DNA synthesis (results not shown).

TABLE II

Effect of fetal bovine serum on the Na^+-H^+ exchange by LLC-PK_{1A}, MDCk4 and BHK cells

Sodium influx through the Na^+-H^+ antiporter was measured as the difference between the $^{22}Na^+$ influx determined in the presence (pH_i 6.0, pH_o 7.5 for LLC-PK_{1A} cells and pH_i 6.0, pH_o 7.0 for $MDCK_4$ and BHK cells) or absence of an outwardly oriented H^+ gradient ($pH_i = pH_o$). Sodium influx in actively proliferating cultures was measured three days after plating. Sodium influx in non-proliferating cells was measured seven days after plating. The results are the means ± S.E. of six different monolayers. * $P < 0.001$.

Cell line	Bovine serum (10%)	Sodium influx (μmol h^{-1} mg DNA^{-1})	
		proliferating	quiescent
LLC-PK_{1A}	no	0.71 ± 0.26	15.84 ± 1.14
	yes	0.90 ± 0.17	13.76 ± 0.77
$MDCK_4$	no	25.07 ± 2.04	0.67 ± 0.09
	yes	39.04 ± 0.91 *	0.93 ± 0.13
BHK	no	1.30 ± 0.10	1.06 ± 0.06
	yes	9.30 ± 0.88 *	1.03 ± 0.10

Polarization of the Na^+-H^+ antiport system in confluent $MDCK_4$ and LLC-PK_{1A} monolayers

To determine whether the different regulatory properties of the Na^+-H^+ antiport system observed in $MDCK_4$ and LLC-PK_{1A} are associated with different topographic localizations, we studied the polarization of these systems in confluent monolayers of $MDCK_4$

TABLE III

Effect of phorbol 12-myristate 13-acetate (TPA) on the Na^+-H^+ exchange by LLC-PK_{1A} and $MDCK_4$ cells

Sodium influx through the Na^+-H^+ antiporter was measured as the difference between the $^{22}Na^+$ influx measured in the presence (pH_i 6.0, pH_o 7.5 for LLC-PK_{1A} cells and pH_i 6.0, pH_o 7.0 for $MDCK_4$ and BHK cells) or absence of an outwardly oriented H^+ gradient ($pH_i = pH_o$). Sodium influx in LLC-PK_{1A} and BHK cells was measured in quiescent cultures seven days after plating while in $MDCK_4$ and BHK cells was measured in actively proliferating cultures three days after plating. The results are the means ± S.E. of six different cultures. * $P < 0.001$.

Cell line	TPA (10^{-6} M)	Sodium influx (μmol h^{-1} mg DNA^{-1})
LLC-PK_{1A}	no	19.09 + 1.91
	yes	19.55 ± 3.00
$MDCK_4$	no	32.48 ± 2.60
	yes	57.79 ± 2.59 *
BHK	no	3.35 ± 0.60
	yes	6.46 ± 1.18 *
Quiescent BHK	no	1.15 ± 0.19
	yes	1.14 ± 0.10

TABLE IV

Polarization of the Na^+-H^+ antiport system in confluent LLC-PK_{1A} and $MDCK_4$ monolayers obtained 7 and 5 days after plating, respectively

Sodium influx was measured in the presence of an outwardly oriented H^+ gradient (pH_i 6.0, pH_o 7.5 for LLC-PK_{1A} cells and pH_i 6.0, pH_o 7.0 for $MDCK_4$ cells) at Na medium concentrations of 1.43 mM and 143 mM for $MDCK_4$ and LLC-PK_{1A} cells, respectively. The transepithelial electrical resistance was 3.47 ± 0.41 kΩ cm^{-2} for $MDCK_4$ monolayers and 97 ± 2 Ω cm^{-2} for LLC-PK_{1A} monolayers. The results are the means ± S.E. of six different monolayers. * $P < 0.001$.

Cell	Influx from	Amiloride (10^{-3} M)	Sodium influx (μmo h^{-1} mg DNA^{-1})
LLC-PK_{1A}	apical	no	70.5 ± 5.0
		yes	33.0 ± 3.0 *
	basolateral	no	73.2 ± 8.2
		yes	80.3 ± 7.8
$MDCK_4$	apical	no	0.55 ± 0.06
		yes	0.34 ± 0.03
	Basolateral	no	3.91 ± 0.17
		yes	0.26 ± 0.05 *

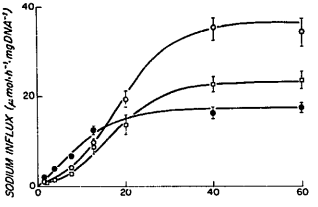


Fig. 4. Sodium influx measured at different extracellular sodium concentrations in LLC-PK_{1A} (closed circles), $MDCK_4$ (open circles) and BHK cells (open squares). Sodium influx through the Na^+-H^+ antiporter was measured as the difference between the $^{22}Na^+$ influx determined in the presence (pH_i 6.0, pH_o 7.5) or absence of an outwardly oriented H^+ gradient (pH_i 7.5, pH_o 7.5). The Na^+ influx in LLC-PK_{1A} cells was measured at confluence in mitotically arrested cells. In $MDCK_4$ and BHK cells, however, Na^+ influx was measured in actively proliferating cultures three days after plating. The results are the means ± S.E. of six different cultures.

and LLC-PK_{1A} cells. Table IV shows that most of the Na^+ influx induced by an outwardly oriented H^+ gradient (pH_i 6.0, pH_o 7.5) and inhibited by 10^{-3} M amiloride occurs from the apical side in LLC-PK_{1A} monolayers. In $MDCK_4$ monolayers, however, the small activity of the Na^+-H^+ antiport that still remains at confluency, occurs at the opposite side (basolateral side) of the monolayers.

Effect of the extracellular Na^+ concentration on the activity of the Na^+-H^+ antiport system in $MDCK_4$, LLC-PK_{1A} and BHK cells

The effect of the extracellular Na^+ concentration on the activity of the antiport system, measured as described before, is shown in Fig. 4. Although the activity of the antiport system increases with increased extracellular Na^+ concentration to reach half-maximal activity at about the same concentration (10 to 18 mM for the three cell lines), the response to sodium is strikingly different in LLC-PK_{1A} cells in comparison with the other two cell lines. LLC-PK_{1A} cells show a hyperbolic response while $MDCK_4$ and BHK show a sigmoid response upon increasing the extracellular sodium concentration. The Hill coefficient calculated for the response observed in both $MDCK_4$ and BHK cells was close to 2.

Effect of extracellular pH on the activity of the Na^+-H^+ antiport system of LLC-PK_{1A} and $MDCK_4$ cells

Fig. 5 shows the activity of the Na^+-H^+ antiport system in confluent LLC-PK_{1A} cells measured at different extracellular pH values and an intracellular pH of 6.0. The results show that the activity of the system increases as the extracellular pH increases, reaching a maximal activity at pH 7.5 and then decreasing at pH 8.0. These results indicate that the optimal extracellular pH for the antiport system observed in LLC-PK_{1A} cells is pH 7.5. Fig. 6 shows the results of a similar study performed in $MDCK_4$ cells. The activity of the Na^+-H^+ antiport system present in $MDCK_4$ cells also increases with the increase in extracellular pH as observed with LLC-PK_{1A} cells. The maximal activity of the antiport system in $MDCK_4$ cells, however, is obtained at an extracellular pH of 7.0 rather than 7.5 as observed with LLC-PK_{1A} cells. Similar results were obtained in BHK cells (results not shown).

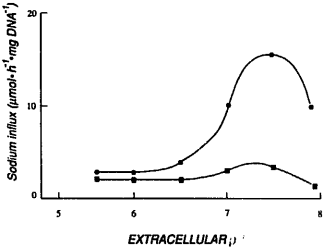


Fig. 5. Effect of extracellular pH on the activity of the Na^+-H^+ antiport in LLC-PK_{1A} monolayers. Sodium influx was measured at different extracellular pH in cells with an intracellular pH of 6.0 (closed circles) or 7.5 (close squares). The results are the means ± S.E. of six different cultures.

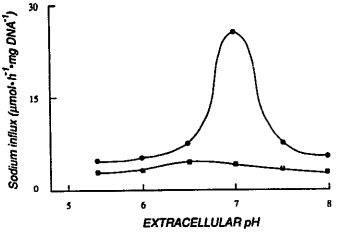


Fig. 6. Effect of extracellular pH on the activity of the Na^+-H^+ antiporter in $MDCK_4$ cells. Sodium influx was measured at different extracellular pHs in cells with an intracellular pH of 6.0 (closed circles) or 7.0 (closed squares). The results are the means ± S.E. of six different cultures.

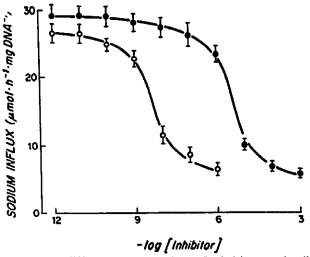


Fig. 8. Effect of different concentrations of ethyl-isopropylamiloride on the sodium influx induced by an outwardly oriented H^+ gradient (pH_i 6.0, pH_o 7.0) in $MDCK_4$ (closed circles) and (pH_i 6.0, pH_o 7.5) $LLC\text{-}PK_{1A}$ cells (open circles). The results are the means ± S.E. of six different cultures.

Effect of different concentration of amiloride on the activity of the Na^+-H^+ antiport system of $MDCK_4$ and $LLC\text{-}PK_{1A}$ cells

Fig. 7 shows the effect of different concentrations of amiloride on the activity of the antiport system measured as sodium influx induced by an outwardly oriented H^+ gradient in $MDCK_4$ and $LLC\text{-}PK_{1A}$ cells. The results show that the antiport system present in $LLC\text{-}PK_{1A}$ cells is more sensitive to the inhibitory effect of amiloride than the system present in $MDCK_4$ cells. Amiloride is 40-times more potent an inhibitor of the Na^+-H^+ antiport system observed in $LLC\text{-}PK_{1A}$ cells (K_i $1.8 \cdot 10^{-7}$ M) than the system present in $MDCK_4$ cells (K_i $7 \cdot 10^{-6}$ M).

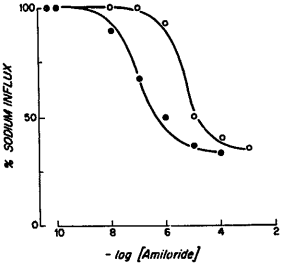


Fig. 7. Effect of different concentrations of amiloride on the Na^+ influx induced by an outwardly oriented H^+ gradient (pH_i 6.0, pH_o 7.0) in $MDCK_4$ (open circles) and (pH_i 6.0, pH_o 7.5) $LLC\text{-}PK_{1A}$ cells (closed circles). The results are the means ± S.E. of six different cultures.

Effect of different concentrations of amiloride and 5-(N-ethyl-N-isopropyl)amiloride on the activity of the Na^+-H^+ antiport system of $MDCK_4$ cells

In order to determine the minimal dose of two well-known inhibitors of the Na^+-H^+ antiport capable of producing maximal or near maximal inhibition of the system, we studied the inhibitory effect of increasing concentrations of amiloride and isopropylamiloride on the Na^+-H^+ exchange by $MDCK_4$ cells. These inhibitors are considered to encompass the range of sensitivities of the antiport system to different amiloride analogs, with amiloride being the less potent and 5-(*N*-ethyl-*N*-isopropyl)amiloride the more potent of the different analogs. The results presented in Fig. 8 show that 5-(*N*-ethyl-*N*-isopropyl)amiloride (K_i $8 \cdot 10^{-9}$ M) as an inhibitor of the Na^+-H^+ antiport system of $MDCK_4$ cells, is greater than 1000-times more potent than amiloride (K_i $7 \cdot 10^{-6}$ M).

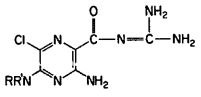
Effect of different analogs of amiloride on the Na^+-H^+ exchange by $LLC\text{-}PK_{1A}$ cells

Table V shows the effect of different amiloride analogs on the Na^+-H^+ exchange by $LLC\text{-}PK_{1A}$ cells. These analogs consisted of those bearing one or two substituents on the 5-amino nitrogen atom of amiloride. Based on the results shown above, the effect of each one of these analogs was studied at a concentration of 10^{-6} M. Amiloride at a concentration of 10^{-3} M was used to show the maximal expected inhibition. The rank for these analogs determined according to their inhibitory potency on the Na^+-H^+ antiport system of $LLC\text{-}PK_{1A}$ cells was: analog V > analog II > analog IV > analog III > analog II.

TABLE V

Effect of one or two substituent on the 5-amino group of amiloride on the Na^+-H^+ exchange by LLC-PK_{1A} cells

Sodium influx was measured in the presence of an outwardly oriented H^+ gradient (pH_i 6.0, pH_o 7.5) in nonproliferating cultures seven days after plating. The results are the means ± S.E. of six different cultures.



Analog (RR'N-) (10^{-6} M)	Amiloride (10^{-3} M)	Sodium influx (μmol h^{-1} mg DNA^{-1})	*n*
None	no	22.50 ± 1.15	(3)
	yes	6.67 ± 0.36	(6)
I.$(CH_3)_2CHN-C_2H_5$	no	8.46 ± 0.31	(6)
II.$(CH_3)_2N-$	no	14.09 ± 0.23	(3)
III.$CH_3-\underset{\displaystyle CH_3}{\overset{\displaystyle CH_3}{C}}-CH_2-N-CH_3$	no	9.61 ± 0.57	(6)
IV.$(CH_3)_3NH-$	no	8.86 ± 0.80	(4)
V.$(CH_3)_2CH-CH_2-N-CH_3$	no	8.15 ± 0.57	(5)

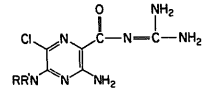
Effect of different analogs of amiloride on the Na^+-H^+ exchange by MDCK$_4$ cells

Table VI shows the inhibitory effect of different amiloride analogs on the Na^+-H^+ exchange by MDCK$_4$ cells. The same analogs were used as in the previous experiment. The experiment was repeated in MDCK$_4$ cells to compare the inhibitory efficacy of the analogs on both cell lines and to determine from these results the differences in the Na^+-H^+ antiport system observed in both cellular systems. The sequence of these analogs determined according to their inhibitory potency is: analog II > analog I > analog III > analog IV > analog V. These results show that there are striking differences in the inhibitory effects of the analogs on the Na^+-H^+ exchange of both cell lines. For instance, analog IV, one of the most effective inhibitors of Na^+-H^+ exchange in LLC-PK_{1A} cells, is the least effective inhibitor in MDCK$_4$ cells. Conversely, analog II, the least effective inhibitor of the Na^+-H^+ exchange in LLC-PK_{1A} cells, is one of the most effective inhibitors in MDCK$_4$ cells.

TABLE VI

Effect of one or two substituent on the 5-amino group of amiloride on the Na^+-H^+ exchange by MDCK$_4$ cells

Sodium influx was measured in the presence of an outwardly oriented H^+ gradient (pH_i 6.0, pH_o 7.0) in proliferating cultures three days after plating. The results are the means ± S.E. of six different cultures.



Analog (RR'N-) (10^{-6} M)	Amiloride (10^{-3} M)	Sodium influx (μmol h^{-1} mg DNA^{-1})
None	no	41.15 ± 1.40
	yes	17.67 ± 1.56
I.$(CH_3)_2CH-N-C_2H_5$	no	18.57 ± 0.90
II.$(CH_3)_2N-$	no	17.96 ± 1.01
III.$CH_3-\underset{\displaystyle CH_3}{\overset{\displaystyle CH_3}{C}}-CH_2-N-CH_3$	no	19.82 ± 1.70
IV.$(CH_3)_3CNH-$	no	21.46 ± 0.63
V.$(CH_3)_2CH-CH_2-N-CH_3$	no	27.11 ± 2.33

Discussion

The present study provides evidence that the Na^+-H^+ antiport system involved in the transepithelial transport of H^+ and the system(s) associated with other cellular functions, such as intracellular pH regulation, cell volume regulation, mitogenic activation, etc., may represent different transport systems. To establish the similarities and differences between these systems, we examined their differential response to cell growth, mitogenic factors, extracellular Na^+ concentration, response to amiloride and several of its analogs and their specific location in the cell membrane (apical vs. basolateral) in several epithelial and nonepithelial cell lines.

Sequence analysis of a recently cloned cDNA encoding a rabbit Na^+-H^+ antiporter [23] shows a high degree of homology with the nucleotide sequence of a cDNA encoding a human growth factor-activable antiporter [45]. This high homology indicates that if there were any functional difference between the systems, the difference(s) will be minimal and difficult to detect.

Several methodological approaches have been used to determine the activity of the antiport system in different cellular systems. These methods determine the activity of the Na^+-H^+ antiporter by measuring the changes in the intracellular pH [17,30] (or its equivalent H^+ release [29]) induced by an inwardly oriented Na^+ gradient; by measuring the changes in the Na^+ influx induced by an outwardly oriented H^+ gradient [30,35,51]; or by a combination of these methods [10,35]. Although, all of these methods give qualitatively similar results, they differ quantitatively to such an extent that it is difficult to perform comparative studies to define the expected very subtle differences between the different Ha +-H^+ antiporters. To avoid this inconvenience, the activity of the Na^+-H^+ antiport system of all cell lines used in the present study was determined using the same experimental approach.

Consistent with previous studies on the development and polarization of the Na^+-H^+ antiport system in LLC-PK_{1A} cells [51], the expression of the system in

this cell line takes place only when there is close cell-to-cell interaction. Cells in active proliferation, as indicated by a large increase in the rate of DNA synthesis, do not express the Na^+-H^+ antiport system. A different result has been obtained in a clone, designated clone 4, isolated from the original LLC-PK_1 cell line [20]. This study indicate that rapidly-proliferating clone 4 LLC-PK_1 cells express Na^+-H^+ exchange activity. It was argued, that the difference between our results (present study and Refs. 11, 50 and 51) and the results obtained with clone 4 of LLC-PK_1 cells was due to the different methodological approaches used to measure the activity of the Na^+-H^+ antiport system. The results obtained with the clone designated LLC-PK_{1B4} isolated from the original cell line, however, indicate that the differences observed may represent differences in cell lines and not necessarily differences in the methodological approach. The totally different response of the antiport system to cell growth observed in two other epithelial cell lines, namely $MDCK_4$ and LLC-PK_{1B4} and the fibroblastic cell line BHK, indicates that the difference observed between these cell and LLC-PK_{1A} cells are not associated with the epithelial or non-epithelial characteristics of the cells. To establish the presence of other differences between the Na^+-H^+ antiport system observed in LLC-PK_{1A} cells and other cell lines, we also studied the response of their respective antiport systems to serum and phorbol esters. The presence of serum induces a complex set of cellular responses that ultimately lead to the initiation of cell proliferation [24]. One of the first events to follow serum stimulation is an increase in activity of the Na^+-H^+ antiport system [16]. The absence of any stimulatory effect of serum on the antiport system of LLC-PK_{1A} sets this system apart from the other two cell lines, in which serum stimulation was clearly observed. Like serum, the tumor promoters phorbol esters have also been shown to initiate a multitude of cellular changes, including stimulation of cell proliferation and differentiation [7]. Although the molecular mechanism(s) that underlies the action of phorbol esters has yet to be identified, recent studies suggest that stimulation of a Na^+-H^+ antiporter may be critical [8,43]. The results obtained with $MDCK_4$ and BHK cells agree with these observations in that both cell lines show a simultaneous increase in the activity of the Na^+-H^+ antiport system and rate of DNA synthesis (results not shown) when incubated in the presence of TPA. Moreover, an important requirement for the stimulatory action of TPA on the antiport system is that the system be pre-stimulated by initiation of cell growth. TPA in confluent and growth-arrested BHK monolayers does not activate Na^+-H^+ exchange, suggesting an indirect effect of phorbol esters on the activation of the antiport system. In LLC-PK_{1A} cells, the presence of TPA affects neither the activity of the Na^+-H^+ antiport system nor the rate of DNA replication. Since the response of the Na^+-H^+ antiport system to cell growth, serum, and phorbol esters in the epithelial cell lines LLC-PK_{1B4} and $MDCK_4$ is similar to the response observed in the fibroblastic cell line BHK and other non-epithelial cell lines, we conclude that, at least from a functional point of view, the presence of Na^+-H^+ exchange in these cells represents the operation of the same antiport system. In contrast, the striking differences between the Na^+-H^+ antiport system of LLC-PK_{1A} cells and the system present in other cell lines indicate that their systems are functionally different or controlled by different regulatory mechanisms. The localization in $MDCK_4$ cells of the Na^+-H^+ antiport system in opposite domains of the cell membrane as compared with the system present in renal proximal tubular cells and LLC-PK_{1A} cells suggests that the system in $MDCK_4$ cells involves a cellular function(s) other than the transepithelial transport of H^+ as observed in the renal proximal tubule. In addition to transepithelial transport, epithelial cells perform other functions common to multiple cellular systems, such as regulation of cytoplasmic pH, control of cell volume, metabolic response to hormones, etc. Although the functional implications of the basolateral localization of the Na^+-H^+ antiport system and its activation by cell proliferation in $MDCK_4$ are unclear, the present study suggests that this system may primarily serve the regulation of cytoplasmic pH, and when required, the mitogenic activation of the cells.

The differences between these systems, appear to go beyond their regulatory mechanisms, as shown by the different kinetics involved in the activation by Na^+, effect of extracellular pH and the different inhibitory effects of amiloride and several of their analogs. Whereas the interaction of external Na^+ with the antiport system in LLC-PK_{1A} cells follows Michaelis-Menten kinetics consistent with a single binding site, similar interactions in $MDCK_4$ and BHK show a sigmoid response curve, suggesting the existence of a second allosteric Na^+ binding site. On the other hand, the activity of the Na^+-H^+ antiporter is not affected by changes in the cell membrane potential, nor does the operation of the system cause measurable changes in the cell membrane potential in LLC-PK_{1A} [10] and $MDCK_4$ cells (results not shown). This electrically silent operation of the Na^+-H^+ antiport system strongly indicates that the $Na^+ : H^+$ coupling ratio in both cell lines is close to 1.0. From these results we conclude that the second Na^+ binding site in $MDCK_4$ and BHK cells could be a modifier rather than a Na^+ transport site. The interaction of external Na^+ with the antiporter in LLC-PK_{1A} cell follows the same Michaelian kinetics observed in brush border vesicles from the renal proximal tubule [1,6]. The sigmoid response to external Na^+, with a Hill coefficient of 2 observed in

$MDCK_4$ and BHK cells, contrasts with the general notion that the stoichiometry of the Na^+-H^+ exchange in other cellular systems including the parent MDCK cell line is 1:1 [17,21,42,44]. Results obtained in other cellular system, however, indicated that operation of the antiporter in a stoichiometric relationship other than a 1:1 is also possible [1,43,47,48]. Moreover, kinetic differences in the effect of extracellular Na^+ on the activity of the Na^+-H^+ antiporter were observed even within the same cellular system (compare Refs. 17 and 48). This observation suggests that the apparent differences in both studies were more the result of the different approach used to measure the activity of the antiporter than the result of true differences between the systems.

The diuretic amiloride inhibits both the conductive Na^+ transport of the so-called tight epithelia and the Na^+-H^+ exchange observed in the renal proximal tubule and other cellular systems [10,17,18,26]. Although at physiological Na^+ concentrations, effective inhibition of Na^+-H^+ exchange requires a rather high amiloride concentration, the diuretic has become a powerful investigative tool for probing the Na^+-H^+ antiport system. In agreement with this concept, our results show that in addition to the different response to cell proliferation, mitogenic factors, external Na^+ and pH and different location in the apical and basolateral membrane, the Na^+-H^+ antiport system of $LLC\text{-}PK_{1A}$ cells is more sensitive to the inhibitory effect of amiloride than the system present in $MDCK_4$ cells. This observation agrees with results obtained in other $LLC\text{-}PK_1$ clone cells suggesting the presence of two pharmacologically different Na^+-H^+ antiporters [19]. Furthermore, our results indicate that appropriate substitution of one or both of the hydrogen atoms on the 5-amino group of amiloride with alkyl or alkenyl groups result in compounds that exhibit increased inhibitory potency on the Na^+-H^+ exchange of $LLC\text{-}PK_{1A}$ and $MDCK_4$ cells. The striking differences observed in the Na^+-H^+ antiport system present in $LLC\text{-}PK_{1A}$ cells and the system present in the other cell lines suggest again the operation of at least two different transport systems. To fully individualize and distinguish these transport systems, they have been designated: (a) 'regulatory' Na^+-H^+ antiport system, the system involved in the intracellular pH regulation, cell volume regulation, mitogenic activation, etc.; and (b) 'Non-regulatory' Na^+-H^+ antiport system, the system localized in the apical membrane of the epithelial cells and involved in the transepithelial transport of H^+.

Acknowledgements

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