Biochimica et Biophysica Acta, 943 (1988) 405–410
Elsevier

BBA 74113

Characterization of Na^+/H^+ exchange in a rabbit corneal epithelial cell line (SIRC)

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(Received 5 April 1988)

Key words: Epithelial cell; SIRC cell line; pH_i intracellular; Sodium ion–proton exchange; (Rabbit cornea)

Continuous intracellular pH (pH_i) measurements were performed in SIRC rabbit corneal epithelial cells using the pH-sensitive absorbance of intracellularly trapped 5(and 6)-carboxy-4′,5′-dimethylfluorescein. Steady-state pH_i in nominally bicarbonate free Ringer's solution averaged 6.87 ± 0.02 (mean ± S.E., $n = 53$). After intracellular acidification induced by the NH_4Cl-prepulse technique, there was a sodium-dependent pH_i recovery towards the normal steady-state pH_i. The initial pH_i recovery rate was a saturable function of extracellular sodium concentration with an apparent K_m for external sodium of about 25 mM and a V_{max} of about 0.28 pH units/min. Virtually no pH_i recovery was observed in the absence of extracellular sodium. Sodium removal during steady state acidified the cells by 0.36 ± 0.05 pH units (mean ± S.E., $n = 13$) within 5 min. There was a dose-dependent inhibition of pH_i recovery after NH_4Cl prepulse by amiloride with an IC_{50} of about 15 μM. Amiloride in a concentration of 1 mM almost completely abolished pH_i recovery. Amiloride (1 mM) applied during steady state induced an intracellular acidification of 0.2 ± 0.03 pH units (mean ± S.E., $n = 7$) within 5 min. These findings suggest that a Na^+/H^+ exchange is present in SIRC rabbit corneal epithelial cells. Na^+/H^+ exchange seems to be the major process involved in pH_i recovery in SIRC cells after an intracellular acid load. Na^+/H^+ exchange also plays a role in the maintenance of steady-state pH_i.

Introduction

Electrolyte and fluid transport across the corneal epithelium plays an important role in the maintenance of corneal transparency [1,2]. It is known from transepithelial studies that active transport of chloride [3], sodium [1] and possibly bicarbonate [4] occurs across the corneal epithelium. Chloride secretion is directed from the stroma to the tear side while species differences have been reported for the net transport direction of sodium [2]. Furthermore, at least in humans, sodium transport seems to be highly sensitive to changes in extracellular and/or intracellular pH [5]. Relatively little is known about the cellular transport mechanisms underlying the transepithelial transport phenomena. In recent years cell culture has provided a useful tool to investigate the basic plasma membrane transport processes involved in epithelial transport in many tissues including epithelial tissues of the eye such as corneal endothelium [6], and ciliary epithelium [7]. In the present study we continuously monitored

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intracellular pH in an established rabbit corneal epithelial cell line, SIRC cells [8], using the pH sensitive absorbance of intracellularly trapped 5(and 6)-carboxy-4′,5′-dimethylfluorescein. We provide evidence for the presence of Na^+/H^+ exchange in cultured SIRC rabbit corneal epithelial cells. Furthermore we have characterized the kinetic properties of this Na^+/H^+ exchange and have investigated its role in pH_i regulation.

Materials and Methods

Cell culture

SIRC cells (Staatens Seruminstitut Rabbit Cornea, *Orycytologus cuniculus*) were obtained at passage 434 from American Type Culture Collection (ATCC CCL 60), Rockville, MD, U.S.A. They were maintained at 37°C in a 5% CO_2/air atmosphere in Eagle's minimum essential medium with Earle's salts supplemented with 10% fetal calf serum, nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were subcultured at a split ratio of 1 : 3 using trypsin/EDTA (0.05/0.02%). Within three days after seeding cells reached confluence and formed a homogeneous monolayer, which could be kept in culture for several weeks without showing overgrowth or multilayer formation. For pH_i experiments cells were seeded on plastic coverslips in Leighton tubes (Costar, Cambridge, MA, U.S.A.) and were used when they had been confluent for at least two days. Cells used in this study were from passages 438–446.

Intracellular pH-measurements

We continuously monitored pH_i using a dual wavelength photometer as described previously [9,10]. In short, the absorbance of 5(and 6)-carboxy-4′,5′-dimethylfluorescein (CDMF) is maximally pH sensitive at about 509 nm, while being pH insensitive at 470 nm (isosbestic point) [11]. Thus, the ratio of absorbance measured simultaneously at 509 nm and 470 nm gives a good estimate for pH_i. These values have been shown to correspond well with values obtained simultaneously with pH-sensitive microelectrodes [12]. Cells grown on plastic cover slips were loaded with dye by a 30 min incubation at room temperature in bicarbonate free saline containing 300 μM CDMF diacetate. At the end of each experiment we performed a calibration of pH_i using the nigericin method [13]. Nigericin, a K^+/H^+-ionophore, was applied in a concentration of 10 μM in a saline solution, whose K^+ concentration (140 mM KCl, 10 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, buffered with 10 mM Hepes, Mops, or Tris to the desired pH) approximates that of the intracellular compartment. Under these conditions pH_i equilibrates with pH_o, and pH_o may be varied to known values. A typical calibration curve is shown at the end of the experiment in Fig. 1. In the following experiments a pH_i scale is shown on the right side of the figures.

Solutions

All experiments were performed in the nominal absence of bicarbonate. The standard bicarbonate free saline contained 151 mM Na^+, 5 mM K^+, 1.7 mM Ca^{2+}, 1 mM Mg^{2+}, 158.4 mM Cl^-, 1 mM SO_4^{2-}, 1 mM $H_2PO_4^-$, 5 mM glucose, 10 mM Hepes, and was adjusted to pH 7.4. Sodium concentration of the solutions was varied by replacing the sodium by *N*-methyl-D-glucamine (NMDG). In solutions designed to change pH_i, an equivalent amount of NaCl was replaced by 20 mM NH_4Cl.

Materials

Cell culture media and supplements were purchased from Biochrom KG, Berlin, Germany, and tissue culture plastic ware was from Nunc A/S, Roskilde, Denmark, CDMF diacetate was obtained from Molecular Probes, Junction City, OR, U.S.A. Nigericin and amiloride were purchased from Sigma, Munich, F.R.G.

Results

Steady-state pH_i

The steady-state pH_i in SIRC cells in standard bicarbonate free saline averaged 6.87 ± 0.02 (mean $\pm$ S.E., $n = 53$). Most animal cells examined so far maintain a cytoplasmic pH of about 6.8–7.2 (Roos and Boron 1981). This value is more alkaline than would be expected if protons were distributed passively across the cell membrane. Thus, transport processes are necessary to extrude H^+ ions against their electrochemical equilibrium.

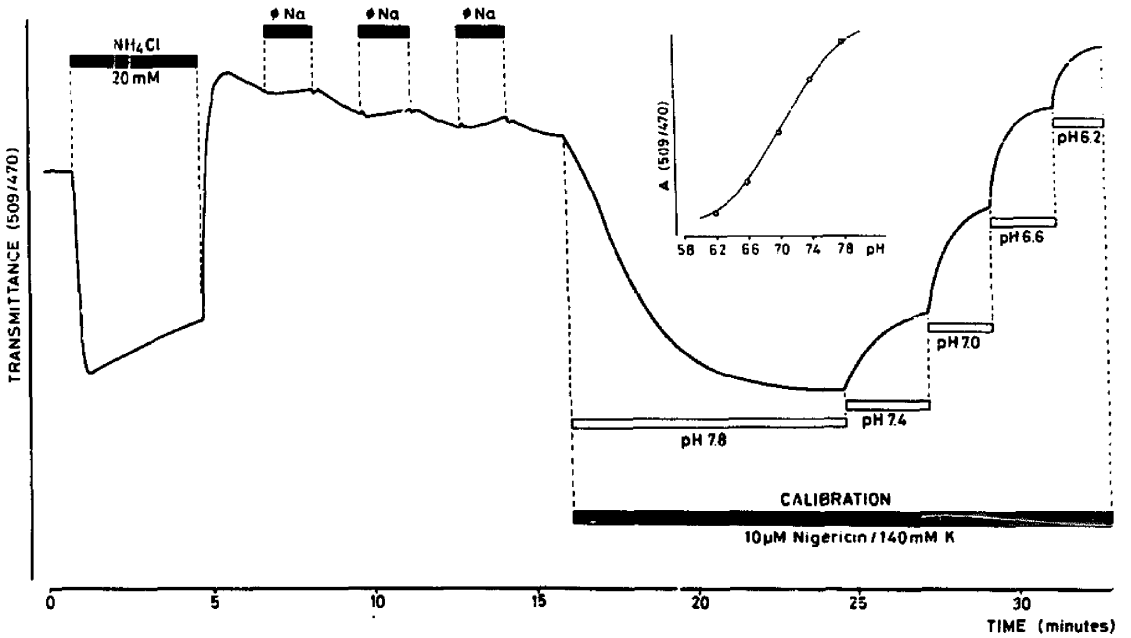

Fig. 1. Effect of sodium removal during pH_i recovery following an acid load. The experiments were performed in the nominal absence of HCO_3^- ($pH_o = 7.4$). Cells were acid-loaded by preincubation for 4 min in 20 mM NH_4Cl, as indicated by the bar above the pH_i trace. Removal of Na^+ (replaced by NMDG) blocked and even reversed pH_i recovery. Note that acidification upon sodium removal becomes more prominent as pH_i approaches steady-state level. At the end of the experiment transmittance was calibrated in terms of pH_i using the nigericin method as described in 'Experimental Procedures'. The inset depicts the calibration curve as absorbance ratio versus pH_i.

pH_i recovery after an intracellular acid load

pH_i recovery was investigated in SIRC cells after imposing an intracellular acid load by means of the NH_4Cl prepulse technique. This technique is a widely used method for acid-loading cells [14,15]. In short, addition of NH_4Cl leads to a rapid rise in pH_i due to influx of NH_3 and its intracellular combination with H^+. This is followed by a slower fall in pH_i due to entry of NH_4^+ down its electrochemical gradient. When the cell is returned to NH_4^+/NH_3-free solution, virtually all the accumulated NH_4^+ gives up a H^+ and exits the cell as NH_3. As a result, pH_i falls far below the initial value and this is followed by a recovery towards normal pH_i as the cell actively extrudes acid equivalents. Typical recordings are shown in Figs. 1 and 2. In the experiment depicted in Fig. 1 sodium was removed during pH_i recovery after NH_4Cl prepulse. pH_i recovery was completely sodium dependent. Moreover, removal of extracellular sodium not only blocked but even reversed pH_i regulation. Reversal of pH_i recovery upon sodium removal becomes more prominent, when pH_i approaches steady state values. Application of 1 mM amiloride, a concentration high

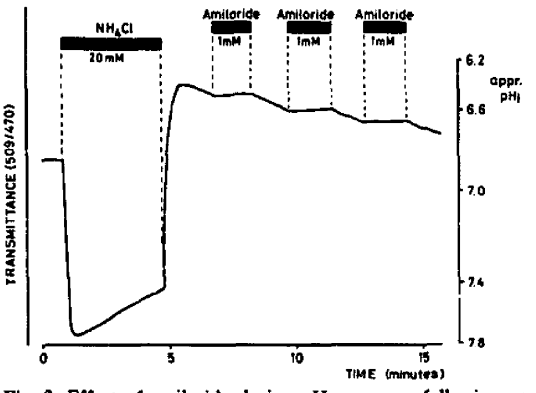

Fig. 2. Effect of amiloride during pH_i recovery following an acid load. Similar experiment as shown in Fig. 1. pH_i recovery after NH_4Cl prepulse was reversibly blocked by the application of 1 mM amiloride.

enough to cause significant inhibition of Na^+/H^+ exchanger in several systems [16], reversibly blocked pH_i recovery after NH_4Cl prepulse (Fig. 2).

Sodium dependence of pH_i recovery

The sodium dependence of pH_i recovery was further investigated. A typical experiment is shown in Fig. 3. The velocity of pH_i recovery increases with increasing extracellular sodium concentration, while virtually no pH_i recovery can be detected in the absence of sodium. Fig. 4A summarizes the results of six experiments similar to that shown in Fig. 3. Initial rates of pH_i recovery were calculated for each sodium concentration used. Kinetic analysis by linear transformation of the data employing the method of Lineweaver-Burk revealed a V_{max} of 0.28 pH units/min with an apparent $K_m = 25$ mM for external sodium (Fig. 4B). Analysis of the same data by Eadie-Scatchard plot revealed a $V_{max} = 0.30$ pH units/min with $K_m = 28$ mM (not shown). Fig. 3 also shows that pH_i was somewhat more alkaline

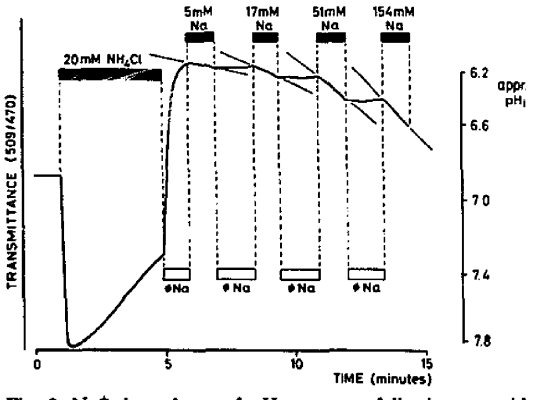

Fig. 3. Na^+ dependence of pH_i recovery following an acid load. The experiment was carried out in the nominal absence of HCO_3^-. Cells were acid-loaded by means of NH_4Cl prepulse. In six similar experiments pH_i after NH_4Cl prepulse averaged 6.24 ± 0.03 (mean $\pm$ S.E.). Na^+ was removed simultaneously with removal of NH_4Cl. In the absence of Na^+ pH_i did not recover from the acid load induced by NH_4Cl prepulse. Subsequently the cells were exposed for short periods (as indicated by the bars) to solutions containing increasing concentrations of Na^+ (6, 17, 51, 154 mM), followed each time by longer intervals of Na^+ removal (in order to reacidify the cells). The initial rates of pH_i recovery were used for kinetic analysis (see Fig. 4).

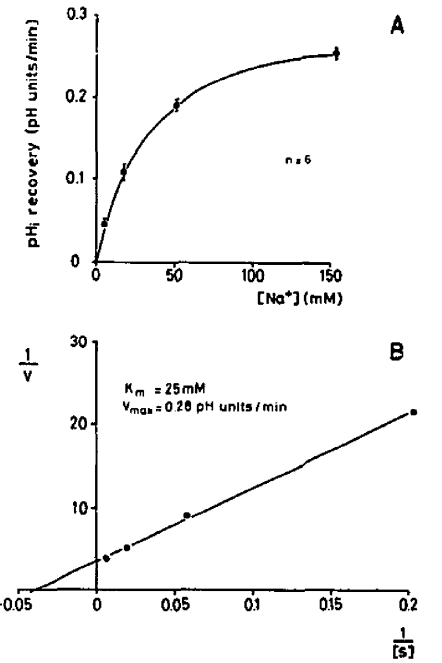

Fig. 4. Kinetic analysis of the Na^+ dependence of pH_i recovery. Results of six experiments similar to that shown in Fig. 3 are summarized. (A) The velocity of pH_i recovery is plotted against the extracellular Na^+ concentration; bars indicate S.E. values ($n = 6$). (B) Linear transformation of the same data employing the method of Lineweaver-Burk.

when 154 mM sodium was added than when 5 mM sodium was added. This could result in an underestimation of the effect of 154 mM sodium, depending on the pH sensitivity of the exchanger [22].

Amiloride sensitivity of pH_i recovery

The dose-dependence of the inhibitory effect of amiloride on pH_i recovery was investigated in a set of experiments summarized in Fig. 5. Standardized NH_4Cl prepulses (4 min, 20 mM NH_4Cl) resulted in a decrease of pH_i to 6.21 ± 0.01 (mean $\pm$ S.E., $n = 36$). Simultaneous with NH_4Cl removal solutions containing 151 mM sodium and amiloride in concentrations from 10^{-7} M up to 10^{-3} M were added. Initial pH_i recovery rates were determined for the various concentrations of amiloride. As demonstrated in Fig. 5, pH_i recovery was inhibited by amiloride in a dose-dependent fashion. The IC_{50} was about 15 μM.

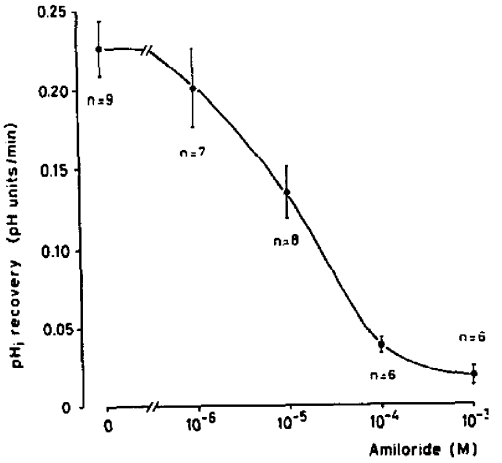

Fig. 5. Amiloride inhibits pH_i recovery after an acid load in a dose-dependent way. A series of experiments is summarized, in which cells were exposed to different concentrations of amiloride as soon as NH_4Cl was removed after a preincubation with 20 mM NH_4Cl for 4 min. NH_4Cl prepulse resulted in a drop of pH_i to 6.21 ± 0.01 (mean $\pm$ S.E., $n = 36$). Initial rates of pH_i recovery were calculated and mean values of six to nine experiments are given. Bars indicate S.E. values. The dose response curve reveals an IC_{50} of about 15 μM at a sodium concentration of 151 mM.

Effects of sodium removal and amiloride on resting pH_i

Replacing extracellular sodium with NMDG, lowered pH_i by 0.36 ± 0.05 pH units (mean $\pm$ S.E., $n = 13$) over a 5 min period (Fig. 6). Readdition of sodium induced a rapid pH_i recovery towards normal steady state pH_i. 1 mM amiloride applied during steady-state conditions reversibly lowered pH_i by 0.20 ± 0.03 pH units (mean $\pm$ S.E., $n = 7$). Sodium removal in the presence of amiloride decreased pH_i by 0.32 ± 0.03 (mean $\pm$ S.E., $n = 7$) pH units.

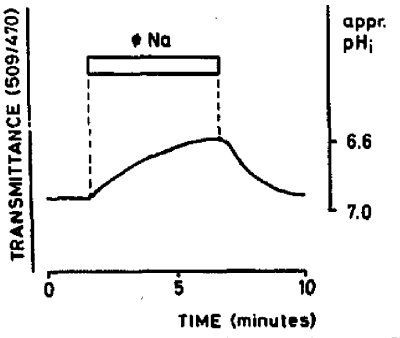

Fig. 6. Effect of sodium removal on steady-state pH_i. The experiment was performed in the nominal absence of HCO_3^-. Under steady-state conditions extracellular sodium was removed for 5 min, which led to an intracellular acidification. Readdition of sodium resulted in rapid pH_i recovery.

Discussion

The SIRC rabbit corneal epithelial cell line has been used by several researchers to investigate properties of corneal epithelial cells under cell culture conditions [17–19]. Working with an established cell line has some advantages, including ease in handling and morphological and biochemical stability after multiple passages. SIRC cells have been shown to proliferate in media containing D-valine substituted for L-valine [17]. According to Gilbert and Migeon [20], this indicates that these cells synthesize the enzyme D-amino-acid oxidase (converting D-valine in the medium into the essential amino acid L-valine), a feature inherent to cells of epithelial origin. Furthermore, SIRC cells have been shown to express similar properties when compared to primary rabbit corneal epithelial cell cultures [17]. Therefore, SIRC cells seem to be a suitable model in which to investigate membrane transport processes in corneal epithelial cells.

Studies of the effects of amiloride on transepithelial transport suggest that Na^+/H^+ exchange might be present in corneal epithelium [21]. In the present study evidence is presented for Na^+/H^+ exchange in SIRC rabbit corneal epithelial cells by means of continuous pH_i recordings: (1) pH_i recovery after an intracellular acid load is sodium dependent and its rate is a saturable function of the extracellular sodium concentration, (2) removal of extracellular sodium during steady state induces intracellular acidification, (3) amiloride inhibits pH_i recovery in a dose-dependent way, (4) application of 1 mM amiloride during steady state decreases pH_i.

Over the past few years an amiloride sensitive Na^+/H^+ exchange mechanism, which is stimulated by acidic pH_i, has been described in many cells. The Na^+/H^+ exchanger has been shown to play an important role in pH_i regulation, volume

regulation, in the response to growth factors, and in transcellular transport of acid equivalents (for reviews see Refs. 22–23). In SIRC cells the apparent K_m of about 25 mM for external sodium and an IC_{50} of about 15 μM for amiloride is in good agreement with values reported for the Na^+/H^+ exchanger in other systems [22]. We have demonstrated that in SIRC cells in the nominal absence of bicarbonate, Na^+/H^+ exchange is the major process responsible for the rapid pH_i recovery after an acute intracellular acid load, since virtually no pH_i recovery can be observed in the absence of extracellular sodium and almost none in the presence of 1 mM amiloride. Furthermore, Na^+/H^+ exchange in SIRC cells seems to function under steady-state conditions, since application of 1 mM amiloride lowers steady state pH_i. Sodium removal during steady state induces a pronounced intracellular acidification, probably because of reversal in the direction of Na^+/H^+ exchange; thus intracellular sodium ions are exchanged for extracellular protons. As expected, the acidifying effect of sodium removal was somewhat reduced by the presence of 1 mM amiloride. However, sodium removal in the presence of amiloride resulted in a larger decrease in intracellular pH than the addition of amiloride alone. It is possible that extracellular amiloride is not as effective in inhibiting Na^+/H^+ exchange when the transport direction is reversed. Moreover, additional effects of sodium removal on intracellular pH (e.g. via Na^+/Ca^{2+} exchange) have to be taken into consideration [9]. We conclude that Na^+/H^+ exchange is not only responsible for pH_i recovery after an acute intracellular acid load but is also involved in the maintenance of steady-state pH_i in SIRC rabbit corneal epithelial cells.

Recent studies in our laboratory in primary cultures of bovine corneal epithelial cells [24] indicate that these cells, too, express a Na^+/H^+ exchange mechanism. Thus, the Na^+/H^+ exchange mechanism seems to be an important cellular transport system not only in rabbit corneal epithelium but also in the corneal epithelium of other species. It is tempting to speculate that Na^+/H^+ exchange in corneal epithelium, besides its pH_i regulating function, may play an important role in transepithelial transport, as has been reported for other epithelia [25,26]. Further studies will be needed to clarify the role of Na^+/H^+ exchange in transcorneal transport.

Acknowledgments

This work has been supported by the Deutsche Forschungsgemeinschaft Grant DFG Wi-328 (11-2) and by a grant from Alcon Research Institute. The expert technical assistance of M. Koch and A. Krolik is gratefully acknowledged.

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