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Cl^- and regulation of pH by MDCK-C11 cells

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Abstract

The interaction between H^+ extrusion via H^+-ATPase and Cl^- conductance was studied in the C11 clone of MDCK cells, akin to the intercalated cells of the collecting duct. Cell pH (pHi) was measured by fluorescence microscopy using the fluorescein-derived probe BCECF-AM. Control recovery rate measured after a 20 mM NH_4Cl acid pulse was 0.136 ± 0.008 pH units/min (dpHi/dt) in Na^+ Ringer and 0.032 ± 0.003 in the absence of Na^+ (0 Na^+). With 0 Na^+ plus the Cl^- channel inhibitor NPPB (10 µM), recovery was reduced to 0.014 ± 0.001 dpHi/dt. 8-Br-cAMP, known to activate CFTR Cl^- channels, increased dpHi/dt in 0 Na^+ to 0.061 ± 0.009 and also in the presence of 46 nM concanamycin and 50 µM Schering 28080. Since it is thought that the Cl^- dependence of H^+-ATPase might be due to its electrogenic nature and the establishment of a +PD (potential difference) across the cell membrane, the effect of 10 µM valinomycin at high (100 mM) K^+ was tested in our cells. In Na^+ Ringer, dpHi/dt was increased, but no effect was detected in 0 Na^+ Ringer in the presence of NPPB, indicating that in intact C11 cells the effect of blocking Cl^- channels on dpHi/dt was not due to an adverse electrical gradient. The effect of 100 µM ATP was studied in 0 Na^+ Ringer solution; this treatment caused a significant inhibition of dpHi/dt, reversed by 50 µM Bapta. We have shown that H^+-ATPase present in MDCK C11 cells depends on Cl^- ions and their channels, being regulated by cAMP and ATP, but not by the electrical gradient established by electrogenic H^+ transport.

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• H^+-ATPase
• Valinomycin
• Cl^-

Introduction

Various intracellular compartments maintain an acidic pH that is necessary for a number of cellular functions, including vesicular trafficking. This is accomplished largely by the active transport of protons (H^+) by the vacuolar type of H^+-ATPase. Active H^+-transport by this pump is electrogenic and generates a voltage gradient by creating an asymmetric distribution of charge across the vesicular membrane. The electrical polarization of this membrane can limit the maximal level of acidification by introducing a positive charge within the vesicle that opposes further movement of protons. The concurrent passive Cl^- movement into vesicular compartments is able to restore

electroneutrality and enables a higher pH gradient to be established (1,2). Several lines of evidence for this interaction has been advanced. An association between the ability to acidify intracellular organelles (endosomes, lysosomes, Golgi apparatus) and the presence of Cl^- ions or Cl^- channels has been observed (3,4).

Dependence of renal H^+-ATPase on Cl^- has also been observed in several studies. The presence of H^+-ATPase in proximal cells was detected by perfusing these tubules *in vitro* in Na^+-free medium and subjecting them to an acid (NH_4Cl) pulse, observing cell pH recovery that was inhibited by n-ethyl-maleimide, an H^+-ATPase blocker (5), or by perfusing them *in vivo* with bafilomycin, a specific H^+-ATPase inhibitor (6). It was shown that the proximal H^+-ATPase was dependent on the presence of Cl^- and on active Cl^- channels in studies on perfused rabbit proximal tubules, where the recovery of cell pH in Na^+-free medium was significantly delayed by perfusion with 5-nitro-2 (3-phenylpropylamine)-benzoic acid (NPPB), a Cl^- channel blocker (7,8). We also observed a significant reduction of bicarbonate reabsorption (a reduction of the rate of H^+ secretion) in the late cortical distal tubule of the rat when apical chloride channels were blocked with NPPB (9).

The interaction of Cl^- ions with H^+-ATPase, however, cannot be explained only by the establishment or dissipation of a potential difference (PD) across cellular and subcellular membranes. It has been shown that the presence of ATP activates not only H^+-ATPase, but also the Cl^- channels connected to it (10); and even after dissipating the PD across endosome membranes with valinomycin, the ATPase still depends on the presence of Cl^- (3).

In the present study, we investigated the relationship between H^+-ATPase and Cl^- ions further by measuring the extrusion of H^+ ions into Na^+-free medium from cultured MDCK cells, more specifically from their C11 clone, which has several properties similar to those of the intercalated cells of the renal distal tubule and collecting duct, using agents known to affect their PD and the activity of their Cl^- channels.

Material and Methods

Cell culture

A subtype (clone) of MDCK cells denominated C11 (MDCK-C11), obtained from Dr. H. Oberleithner (Department of Physiology, University of Münster, Germany), was used (11). These cells were used from passage 74 to passage 82. Cultures were maintained in minimum essential medium with Earle's salts, nonessential amino acids, and L-glutamine (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 26 mM $NaHCO_3$, 100 IU/mL penicillin and 100 µg/mL streptomycin. Cells were grown at 37ºC in 95% humidified air and 5% carbon dioxide, pH 7.4, in a CO_2 incubator (Lab-Line Instruments Inc., Melrose Park, IL, USA). The medium was changed three times a week and the cells were split once a week. The cells were harvested with trypsin and then seeded onto sterile permeable filters (Costar, Cambridge, MA, USA) with 12 mm in diameter and pore size of 3 µm, and then incubated for 48-72 h in the same medium until they reached confluence.

Measurement of pHi by fluorescence microscopy

Cell pH (pHi) was measured using the fluorescent probe 2',7'-biscarboxyethyl-5,6-carboxyfluorescein (BCECF) (12,13). Cells grown to confluence on permeable filters were loaded with the dye by exposure for 20 min to 12 µM BCECF-AM in the control solution (solution 1, Table 1). The acetoxymethyl ester of BCECF enters the cells and is rapidly converted to the anionic free acid form (4 negative charges) by intracellular

esterase. Following the loading period the filters covered with confluent cell monolayers were placed in a thermo-regulated chamber mounted on an inverted epifluorescence microscope (Nikon, Diaphot model TMD, Tokyo, Japan). In all experiments, the solutions were the same on both surfaces of the cell layer. The use of permeable filters permits adequate access of the solutions to both cell surfaces. The filters were rinsed with the control solution to remove the solution containing BCECF. The area measured under the microscope had a diameter of 260 µm and contained up to 200 cells. All experiments were performed at 37°C.

The cells were alternately excited at 440 or 495 nm with two 150-W xenon lamps illuminating the measurement chamber via a bifurcated fiber optic glass guide and the fluorescence emission was monitored at 530 nm with a photomultiplier-based fluorescence system (PMT-4000, Georgia Instruments, Roswell, GA, USA) at 5-s intervals. At the end of an experiment, the BCECF signal was calibrated using the high K^+-nigericin method (13,14). pHi was set approximately equal to pHo by exposing the cells to a solution containing 130 mM KCl, 20 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, and 10 µM nigericin, at different pH values. The 495/440 ratio was measured at different pHo, and the resulting plot of 495/440 ratios *vs* pH yielded a calibration curve which was used to convert the fluorescence ratios (495/440 nm) to pHi.

To analyze the functional activity of the H^+ transporters the intracellular alkalinization following an acute acid load due to a 20 mM NH_4Cl pulse was determined (solution 2), allowing us to calculate the initial rate of pHi recovery (dpHi/dt, pH units per min) in the presence of different solutions from the first 2 min of the recovery curve by linear regression analysis in the presence of Na^+. In the absence of Na^+, recovery started 2-3 min after returning to the NH_4Cl-free solution, when recovery rates were measured over a 2-min period.

Solutions and reagents

The composition of the solutions used in the present study is shown in Table 1. The osmolality of the solutions ranged from 295 to 305 mOsm. Na^+ concentration in nominally Na^+-free solutions was less than 1 mEq/L. Concanamycin A, 8-bromo-cAMP, valinomycin and nigericin were obtained from Sigma, St. Louis, MO, USA and Schering 28080 (Sch 28080) was obtained from Schering-Plough (Liberty Corner, NJ, USA). BCECF-AM and Bapta-AM were obtained from Molecular Probes Inc., Junction City, OR, USA. NPPB was a gift from Dr. R. Greger, Freiburg, Germany. All other chemicals were of analytical grade.

Statistical analysis

Data are reported as means ± SEM. Statistical comparisons were made by analysis of variance followed by the Student-Newman-Keuls contrast test, with the level of significance set at $P < 0.05$.

Results

The MDCK-C11 cells studied in the present investigation, which have properties similar to collecting duct intercalated cells, had a mean pH of 7.19 ± 0.02 (N = 76) in control sodium Ringer solution (Table 1, solution 1). In the experimental groups, care was taken to use cell pH series in which the pH obtained after an acid pulse reached similar values (of the order of 6.4-6.6). Figure 1 illustrates the recovery of cell pH after an ammonium pulse in the absence of Na^+ plus 10 µM NPPB, a Cl^- channel blocker, compared to the curve in Na^+ Ringer (Figure 1A), and in the absence of Na^+ (0 Na^+) and Cl^- (solution 5) compared to 0 Na^+ plus Cl^- (Figure 1B). In the presence of NaCl after cellular acidification, cell pH was recovered

at a rate of 0.136 ± 0.008 pHi units/min (N = 15). At 0 Na^+, with pH recovery starting after only about 2-3 min of the pulse, this rate was 0.032 ± 0.003 pHi units/min (N = 10), and at 0 Na^+/0 Cl^-, the rate was 0.013 ± 0.005 pHi units/min (N = 11). When NPPB was included in the 0 Na^+ experiment, the recovery was 0.014 ± 0.0007 (N = 6) pHi/min, a value similar to that observed during superfusion with 0 Na^+/0 Cl^-. The mean values for these groups are given in Figure 1C. It can be seen that the recovery of pHi at 0 Na^+ was significantly slower than in sodium Ringer solution ($P < 0.001$), and that the recovery was significantly slower in both 0 Na^+/0 Cl^- and 0 Na^+ + NPPB than in 0 Na^+ alone ($P < 0.01$).

Since the reduction of cell pH recovery observed at low chloride levels or after blocking Cl^- channels has been attributed to shunting of the PD caused by electrogenic H^+ secretion (e.g., by vacuolar H^+-ATPase) by the Cl^- anions, we investigated the effect of abolishing cell PD by performing the experiments in the presence of 10 µM valinomycin, a potassium ionophore known to reduce transmembrane PD (15) at high external potassium levels (100 mM K^+ substituting the cation of solutions 1 or 3). Figure 2 shows the mean values of dpHi/dt (cell pH units/min) for several of these experimental groups. First, valinomycin/high K^+ was used in 45 mM Na^+-containing solutions. In this experiment, the addition of valinomycin/K^+ significantly increased dpHi/dt from 0.124 ± 0.01 (N = 5) to 0.233 ± 0.018 (N = 9). The cause of this increase is not clear. It might have been due to the low PD, which might favor Na^+ extrusion, lower cell Na^+, and a more favorable gradient for Na^+ influx via the electroneutral exchanger. A similar effect of valinomycin on Na^+/H^+ exchanger activity has been reported in a different cell model (cheek cells) (16). On the other hand, no significant difference was found when comparing valinomycin/K^+ with 0 Na^+, with dpHi/dt being 0.032 ± 0.003 for 0 Na^+ (N = 10) and 0.030 ± 0.003 for 0 Na^+ + valinomycin (N = 7). Also, no significant difference was observed when comparing the solution containing 10 µM NPPB (0.014 ± 0.001; N = 6) to the solution containing NPPB + valinomycin (0.018 ± 0.003; $P > 0.05$; N = 7). Moreover, in the absence of Na^+ + valinomycin but with control levels of K^+ (5 mM), dpHi/dt was 0.034 ± 0.008 pH units/min (N = 5), a value not different from that observed with 100 mM K^+. The cell membrane potentials found in this cell line (C11 MDCK) have been reported by Gekle et al. (17) and similar values were expected to be present in our experiments, as shown in Table 2. These data suggest that membrane PD was not an important factor responsible for the effect of Cl^- on dpHi/dt.

One of the important regulators of chloride channels, especially in the intestinal mucosa, but also in renal tubules, is cAMP (18-20). In subsequent experiments, 10 µM 8-Br-cAMP, a membrane-permeant form of cAMP, was added to C11 cells incubated in 0 Na^+ solution (see Figure 3). Under these conditions, dpHi/dt increased significantly

Table 1. Composition of the solutions used in the present experiments.

	Solution 1 (control)	Solution 2 (NH_4Cl)	Solution 3 (0 Na^+)	Solution 4 (0 Cl^-)	Solution 5 (0 Na^+/0 Cl^-)
NaCl	145	125	-	-	-
KCl	5.0	5.0	5.0	-	-
NaH_2PO_4	1.0	1.0	-	1.0	-
Na_2SO_4	1.0	1.0	-	1.0	-
$CaCl_2$	1.8	1.8	1.8	-	-
HEPES	30	30	30	30	30
$MgCl_2$	1.0	1.0	1.0	-	-
NMDG	-	-	147	-	147
NH_4Cl	-	20	-	-	-
Glucose	10	10	10	10	10
Na^+ gluconate	-	-	-	145	-
K^+ gluconate	-	-	-	5.0	5.0
Ca^{2+} gluconate	-	-	-	7.0	7.0
Mg^{2+} gluconate	-	-	-	1.0	1.0
pH	7.4	8.0	7.4	7.4	7.4

Concentration is given in mM. HCl or NaOH (0.1 N) were used in solutions 1 and 2 to titrate to the appropriate pH. N-methyl d-glucamine (NMDG) (0.1 M) was used for titration in Na^+-free (0 Na^+) solutions (solutions 3 and 5) and D-gluconic acid was used in the Cl^--free (0 Cl^-) solutions (solutions 4 and 5).

from 0.032 ± 0.003 (N = 6) at 0 Na^+ to 0.061 ± 0.009 (N = 9) with 8-Br-cAMP. When 46 nM concanamycin, a specific blocker of vacuolar H^+-ATPase, was added to the 0 Na^+ solution, dpHi/dt fell significantly to 0.020 ± 0.003 (N = 6), but 8-Br-cAMP still increased pH recovery significantly to 0.034 ± 0.005 (N = 8). When 50 µM Sch 28080, an inhibitor of H^+-K^+-ATPase, was added to the 0 Na^+ solution, dpHi/dt did not change significantly compared to control 0 Na^+, with a value of 0.033 ± 0.004 (N = 9), but the addition of 8-Br-cAMP still increased pH recovery significantly to 0.046 ± 0.003 (N = 10) pH units/min. These data indicate a marked participation of H^+-ATPase in pH recovery in these cells, but a minor participation of H^+-K^+-ATPase, lower than that reported in a previous paper (12). However, 8-Br-cAMP stimulated pH recovery after an acid load in the three situations. When both concanamycin and Sch 28080 at the concentrations given above were added to C11 cells incubated in 0 Na^+ Ringer, dpHi/dt fell to 0.010 ± 0.0091 (N = 6), and when 10 µM 8-Br-cAMP was further added, a dpHi/dt of -0.026 ± 0.023 (N = 4) was obtained, with neither value differing significantly from 0 (P > 0.10).

The role of chloride depletion of C11 cells prior to the addition of 8-Br-cAMP was investigated in subsequent experiments. Figure 4 presents the mean values of dpHi/dt in experiments at 0 Na^+ with and without the addition of 10 µM 8-Br-cAMP in the absence or presence of chloride depletion (solutions 4 or 5). Control values in the presence of Na^+ were also included. The 0 Na^+ +/- 8-Br-cAMP data without depletion are those given above. In the Cl^- depletion group, before measuring basal pH values the cells were incubated for 10 min in 0 Cl^- solutions. Here, dpHi/dt was 0.041 ± 0.003 in 0 Na^+ (N = 5) and 0.036 ± 0.004 (N = 8) after the addition of 8-Br-cAMP, without a significant difference between these groups. In chloride depletion, the effect of 8-Br-cAMP on cell pH recovery due to vacuolar H^+-ATPase appeared to have been abolished due to the absence of Cl^- fluxes across the channels present in the cell membranes of C11 cells.

An interaction of cystic fibrosis trans-

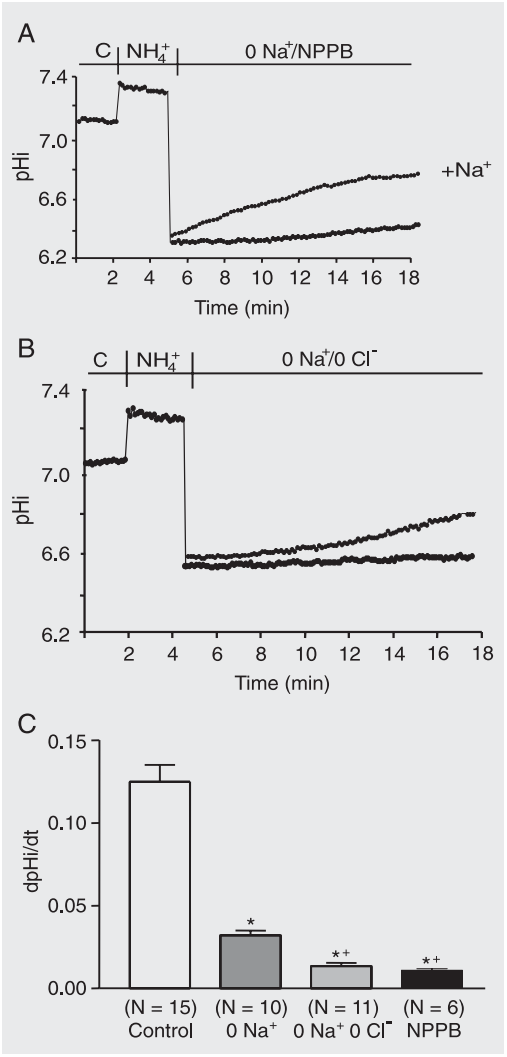

Figure 1. Records of cell pH under control (C), ammonium pulse (NH_4) and experimental conditions in MDCK-C11 cells. *A*, Superfusion with 0 Na^+ Ringer solution (solution 3) + 10 µM NPPB. For comparison, + Na^+ was superfused with Na^+ Ringer (solution 1). *B*, Superfusion with 0 Na^+/0 Cl^- Ringer solution (solution 5) compared to 0 Na^+ alone (solution 3). *C*, Mean initial rate of pHi recovery following acute intracellular acidification in MDCK-C11 cells. Each bar represents the mean ± SEM rates of cell pH recovery (dpHi/dt, pH units/min) under different experimental conditions (control Na^+ Ringer, 0 Na^+ Ringer, 0 Na^+/0 Cl^- Ringer, and 0 Na^+ Ringer plus 10 µM NPPB). NPPB = 5-nitro-2 (3-phenylpropylamine)-benzoic acid. *P < 0.001 *vs* control. +P < 0.01 *vs* 0 Na^+ (ANOVA).

Table 2. pH recovery rates after an acid pulse at different external [K^+] concentrations in the presence and absence of valinomycin.

$[K^+]_e$	Valinomycin	dpH/dt	Approximate Vm*
5 mM	0	0.032 ± 0.003 min^{-1}	~ -35-40 mV
5 mM	10 µM	0.034 ± 0.008	E_K (~ -90 mV)
100 mM	10 µM	0.030 ± 0.003	~ 4.8 mV

*Data taken from Ref. 17.

membrane conduction regulator (CFTR) channels with ATP has been observed; particularly, exit of ATP from cells via these channels has been reported (21). Purinergic receptors have been detected in C11 cells (22). In addition, it was shown that extracellular ATP stimulates Cl^- secretion while increasing cell Ca^{2+} in both renal and digestive epithelial cells (20,23). The activation of Cl^- conductance may modulate H^+-ATPase in C11 cells. We have investigated this possibility by measuring the recovery of cell pH during administration of 100 µM ATP to Na^+ Ringer or 0 Na^+ Ringer solution. dpH/dt was 0.146 ± 0.007 (N = 18) in Na^+ Ringer, and, 0.063 ± 0.004 (N = 6) in Na^+ Ringer + 100 µM ATP ($P < 0.001$). dpHi/dt was 0.035 ± 0.002 (N = 5) in control 0 Na^+ Ringer, and 0.015 ± 0.001 (N = 7) in Ringer with 100 µM ATP ($P < 0.01$). In order to detect a role for cell Ca^{2+} in this effect, dpHi/dt was measured in the presence of 50 µM Bapta-AM, an intracellular Ca^{2+} chelator. The addition of Bapta to 0 Na^+ Ringer did not affect dpHi/dt significantly (0.031 ± 0.004, N = 4, $P > 0.05$), but Bapta + ATP led to the recovery of dpHi/dt to values not significantly different from 0 Na^+ alone (0.040 ± 0.0095, N = 5, $P > 0.05$), thus abolishing the effect of ATP on pH recovery (see Figure 5). This finding suggests mediation of Ca^{2+} in the effect of ATP on H^+ transport.

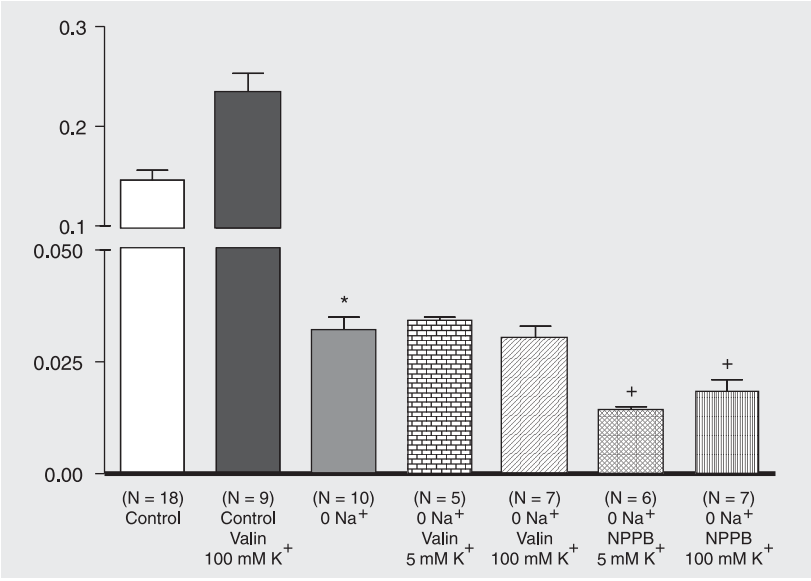

Figure 2. Effect of abolishing cell potential difference (PD) on the observed reduction of cell pH after blocking Cl^- channels. Means ± SEM of dpHi/dt (pH units/min) in control (Na^+ Ringer) and 0 Na^+ Ringer during superfusion with 10 µM valinomycin (valin)/100 mM K^+ and/or 0 Na^+/10 µM 5-nitro-2 (3-phenylpropylamine)-benzoic acid (NPPB). *P < 0.05 *vs* control; +P < 0.05 *vs* 0 Na^+ (ANOVA).

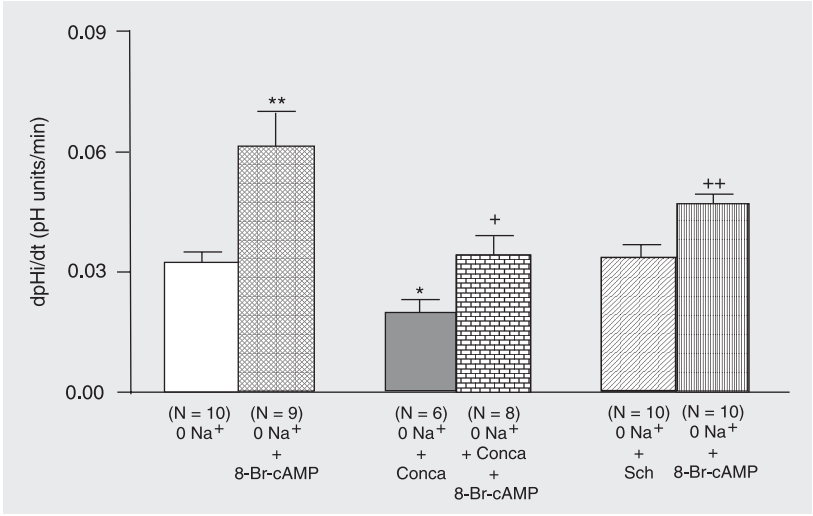

Figure 3. Effect of 10 µM 8-Br-cAMP, a membrane-permeant form of cAMP, on the mean initial rate of pHi recovery in C11 cells incubated in 0 Na^+ solution. Mean ± SEM dpHi/dt during superfusion with 0 Na^+ Ringer containing 10 µM 8-Br-cAMP, 46 nM concanamycin (Conca), an H^+-ATPase blocker, and/or 50 µM Schering 28080 (Sch), an H^+/K^+-ATPase inhibitor. *P < 0.05 *vs* control (0 Na^+). **P < 0.01 *vs* 0 Na^+. +P < 0.05 *vs* concanamycin. ++P < 0.05 *vs* Sch 28080 (unpaired t-test).

Discussion

The present paper investigated the role of Cl^- ions in H^+ ion transport across plasma membranes of a clone of cultivated MDCK cells, C11 cells, that were obtained by Gekle et al. (11) and characterized as a model for renal-collecting duct intercalated cells. Experiments from our laboratory have shown that these cells possess Na^+/H^+ exchangers, H^+-ATPase and H^+-K^+-ATPase in their membranes, the latter transporters being demonstrated during experiments in 0 Na^+ solutions (12). Figure 3 shows that in the present series of experiments, treatment of the cells with Sch 28080 did not reduce dpHi/dt significantly when compared to 0 Na^+, indicating that H^+/K^+-ATPase did not have a sig-

nificant participation in our experiments. Nevertheless, cAMP stimulation of H^+ transport in the presence of the H^+/K^+-ATPase blocker Sch 28080 yielded a lower rate than when cAMP was applied to 0 Na^+ alone; thus, a role of H^+/K^+-ATPase in our experiments cannot be entirely excluded. The present experiments confirmed the role of Cl^- in H^+ secretion by vacuolar H^+-ATPase in Na^+-free solutions that had been previously shown in wild-type MDCK cells (24) and in the S3 segment of rabbit proximal tubules (7), indicating that intercalated cells (C11 cells) are an important site for the expression of H^+-ATPase in the distal nephron.

The interaction of H^+-ATPase and Cl^- channels has also been observed by other investigators. Colocalization of H^+-ATPase and ClC5 channels has been observed in renal cells by immunocytochemical analysis using monoclonal antibodies against these transporters. This colocalization was seen in proximal tubules and in collecting duct intercalated cells, inside subcellular membrane vesicles. The presence of Cl^- has been shown to support mechanisms leading to apical membrane insertion of these ATPases, as well as to filtered protein reabsorption (25-27). ClC3 and CFTR channels have also been implicated in this mechanism (2,28). CFTR channels are known to be important for Cl^- secretion in intestinal as well as renal epithelia (29). These channels transport ATP and are known to regulate the activity of other channels, including Cl^-, Na^+ (ENaC) and K^+ channels (30,31).

It has been widely accepted that chloride ions moving along chloride channels act to dissipate the electrical gradient established by the electrogenic transport of H^+ ions into subcellular vesicles (endosomes, lysosomes, Golgi vesicles) (4,32-34). However, evidence for different mechanisms for the coupling of H^+-ATPase and Cl^- has been obtained. Kaunitz et al. (3) have shown that the acidification of renal medullary endosomes depends on the presence of Cl^- ions, but the shunting of the PD created by H^+-ATPase by valinomycin, a potassium ionophore, at high external K^+, did not affect the Cl^- dependence of H^+ transport. We have followed up this question by incubating C11 cells in a medium containing valinomycin and 5 or 100 mM external K^+, and by measuring the recovery of cell pH after an acid ammonium

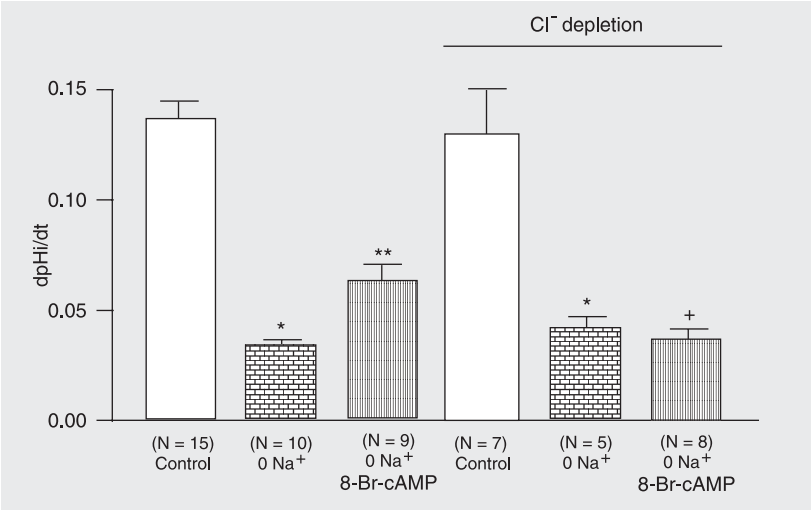

Figure 4. Effect of 10 µM 8-Br-cAMP on dpHi/dt in controls or 0 Na^+ Ringer solution in normal and Cl^--depleted C11 cells. *P < 0.01 *vs* control Na^+. **P < 0.05 *vs* 0 Na^+; $^+$P > 0.05 *vs* 0 Na^+ (ANOVA).

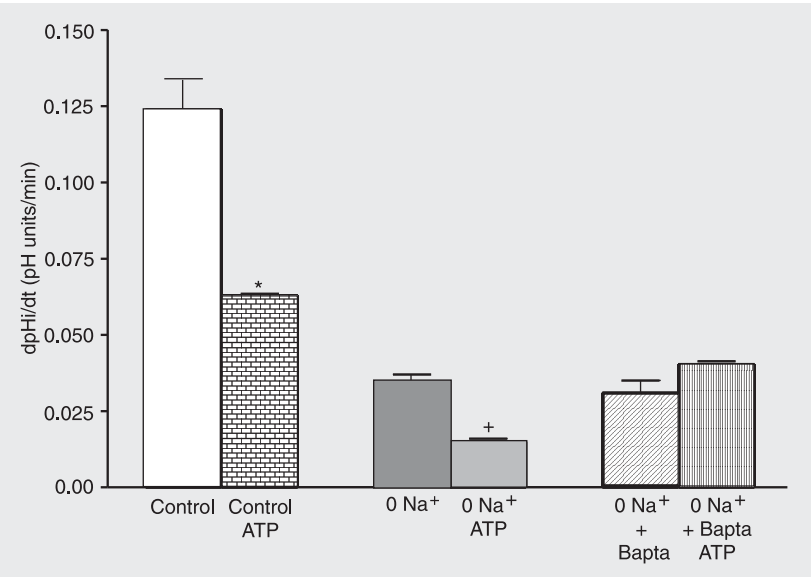

Figure 5. Effect of 100 µM ATP on dpHi/dt in C11 cells superfused with Na^+ or 0 Na^+ Ringer solution, and effect of Bapta-AM during superperfusion with 0 Na^+ solution. *P < 0.01 *vs* control Na^+; $^+$P < 0.01 *vs* 0 Na^+ (unpaired *t*-test).

pulse. As seen in Figure 2, the presence of valinomycin did not alter dpHi/dt measured at 0 Na^+ in the presence of NPPB. Under these experimental conditions, the PD across the cell membrane was expected to fall markedly. Assuming an intracellular K^+ of 120 mM and an extracellular K^+ of 100 mM, one obtains a cell PD of -4.8 mV by the Nernst equation when the permeability of K^+ is determinant. This finding indicates that electrical shunting is not the main mechanism of the functional coupling between H^+ and Cl^- transport in C11 cells, as had been suggested by Kaunitz et al. (3) for renal medullary endosomes. Some other form of coupled H^+/Cl^- transport should be invoked, such as that suggested for a group of red pigments produced by microorganisms, called prodigiosins, that inhibit lysosomal acidification and uncouple H^+-ATPase H^+ transport without affecting ATP hydrolysis (14,35). These molecules are thought to be H^+/Cl^- symporters and function as H^+-ion ionophores that may lead to dissipation of pH gradients across subcellular vesicle membranes. On the other hand, in the present experiments we observed the transfer of a relatively small amount of H^+ across the whole cell membrane, a situation quite different from that observed in endosomes, where the density of H^+-ATPases is certainly much higher. Nevertheless, in both preparations the dependence of H^+ transport on Cl^- is apparent.

The role of cAMP in the regulation of intestinal chloride secretion via CFTR channels has been widely acknowledged, and has also been detected in the distal nephron (cortical-collecting duct) (19). In our experiments (see Figures 3 and 4), the addition of 8-Br-cAMP, a membrane permeant form of cAMP, to the incubation medium stimulated the recovery of cell pH in 0 Na^+ solution, which could suggest that stimulating Cl^- flux through cAMP-sensitive Cl^- channels could also affect the possibly co-localized H^+-ATPase. In our experiments, 8-Br-cAMP stimulated H^+ extrusion in Na^+-free solutions both after blocking H^+-K^+-ATPase with Sch 28080 and H^+-ATPase with concanamycin, suggesting that both of these H^+-transporting molecules may be stimulated by cAMP. This is compatible with the Cl^-/H^+-ATPase interaction discussed above and agrees with the mechanisms proposed for the action of cAMP phosphorylating CFTR channels, thereby destabilizing their closed state and thus increasing the membrane conductance to Cl^- (36), which could also lead to activation of H^+-ATPase. It is not clear if cAMP acts directly on these transporters or via activation of Cl^- channels. It has been proposed that the action of CFTR on H^+-ATPase might be indirect, with CFTR activating other Cl^- channels such as ClC5 which in turn would increase membrane conductance for Cl^-, with this increase stimulating H^+-ATPase.

We have also shown that in cells preincubated at 0 Cl^- no effect of 8-Br-cAMP on cell H^+ extrusion was found, which supports the view that increases of Cl^- fluxes are an integral part of the activation of H^+-ATPase. On the other hand, we have demonstrated before that incubation of C11 cells in 0 Cl^- reduces the volume of these cells, a process that may stimulate ion transport mechanisms transferring ions into them, thus recomposing their volume (13). This process could modify the rate of H^+ extrusion from the cells. However, it has been proposed that, after hypotonic shock, a regulatory volume decrease would cause opening of CFTR and their parallel ATP channels, releasing ATP into the extracellular medium, which, by interacting with P2 receptors, might increase the probability of Cl^- channel opening (37,38). This mechanism would not be active in the volume decrease caused by Cl^- depletion, turning volume regulation an improbable cause for the activation of dpHi/dt during this depletion. In addition, in the study cited above we had also shown that influx of NH_4^+ via the Na^+/K^+ ATPase is stimulated in 0 Cl^- medium, probably due to

the volume reduction observed in this medium. This process could participate in the change in cell alkalinization; however, the magnitude of dpHi/dt in 0 Na^+ medium under Cl^- depletion was similar to that in Cl^- Ringer solution, suggesting that no major modifications in H^+ extrusion occurred under these experimental conditions.

The stimulation of H^+-K^+-ATPase by cAMP observed in our experiments was unexpected. However, a similar effect was observed for gastric glands, where it was shown that this ATPase is phosphorylated via cAMP/PKA, causing fusion of tubular vesicles with the apical membrane, which would be compatible with a stimulating effect of cAMP on H^+-K^+-ATPase in our preparation (39). On the other hand, in the present series of experiments, the participation of H^+/K^+-ATPase in H^+ extrusion was minor, as shown in Figure 3; the reduction of dpHi/dt upon the addition of Sch 28080 to the medium was small, indicating that H^+-ATPase is the major component of H^+ transport under 0 Na^+ conditions.

In contrast to the effect of cAMP on H^+-ATPase, which was stimulatory, extracellular ATP inhibited H^+ extrusion from C11 cells via this transporter. The stimulating effect of extracellular ATP on Cl^- secretion or conductance has been reported (see Results). In one of these studies, performed in *Necturus* gallbladder, activation of Cl^- conductance was shown to depend on cAMP/PKA, and not on Ca^{2+} (20), while in the other, performed in renal collecting duct cells, treatment with Bapta reduced Cl^- current significantly (23). In our study, H^+ extrusion via H^+-ATPase was stimulated by cAMP and reduced by ATP in a Ca^{2+}-dependent manner. It should be kept in mind, however, that there are at least four different purinergic P1 receptor isoforms and an even greater number of P2 isoforms that may interact with ATP, so that a direct interaction of ATP with one or more of the different H^+ transporters cannot be excluded (40). This review also shows that transient activation and delayed inhibition of NKCC in ATP-treated C11-MDCK cells is mediated by Ca^{2+}/CaM-dependent protein kinase II and by Ca^{2+}-independent signaling triggered by apical P2Y(2) and basolateral P2Y(1) receptors, respectively, demonstrating a possible interference of Ca^{2+} with the effect of ATP on cell membrane H^+ transport.

The present data indicate that MDCK-C11 cells, that have many properties of collecting duct intercalated cells, possess in their plasma membranes H^+-ATPases responsible for part of the recovery of their pH after an acid pulse. This mechanism depends on Cl^- for its activation, but cell PD is not an important factor for the maintenance of its activity, since its abolition by valinomycin/high K^+ did not affect the function of H^+-ATPase. Other regulatory factors such as cAMP, ATP and chloride depletion were also investigated in the present study.

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