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H^+ ATPase and Cl^- Interaction in Regulation of MDCK Cell pH

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Abstract. MDCK cells display several acid-base transport systems found in intercalated cells, such as Na^+-H^+ exchange, H^+–K^+ ATPase and Cl^-/HCO_3^- exchange. In this work we studied the functional activity of a vacuolar H^+-ATPase in MDCK cells and its chloride dependence. We measured intracellular pH (pHi) in monolayers grown on glass cover slips utilizing the pH sensitive probe BCECF. To analyze the functional activity of the H^+ transporters we observed the intracellular alkalinization in response to an acute acid load due to a 20 mM NH_4^+ pulse, and calculated the initial rate of pHi recovery (dpHi/dt). The cells have a basal pHi of 7.17 ± 0.01 (n = 23) and control dpHi/dt of 0.121 ± 0.006 (n = 23) pHi units/min. This pHi recovery rate is markedly decreased when Na^+ was removed, to 0.069 ± 0.004 (n = 16). It was further reduced to 0.042 ± 0.005 (n = 12) when concanamycin 4.6×10^{-8} M (a specific inhibitor of the vacuolar H^+-ATPase) was added to the zero Na^+ solution. When using a solution with zero Na^+, low K^+ (0.5 mM) plus concanamycin, pHi recovery fell again, significantly, to 0.023 ± 0.006 (n = 14) as expected in the presence of a H^+–K^+-ATPase. This result was confirmed by the use of 5×10^{-5} M Schering 28080. The Na^+ independent pHi recovery was significantly reduced from 0.069 ± 0.004 to 0.042 ± 0.004 (n = 12) when NPPB 10^{-5} M (a specific blocker of Cl^- channels in renal tubules) was utilized. When the cells were preincubated in 0 Cl^-/normal Na^+ solution for 8 min. before the ammonium pulse, the pHi recovery fell from 0.069 ± 0.004 to 0.041 ± 0.007 (n = 12) in a Na^+ and Cl^- free solution. From these results we conclude that: (i) MDCK cells have two Na^+-independent mechanisms of pHi recovery, a concanamycin sensitive H^+-ATPase and a K^+ dependent, Schering 28080 sensitive H^+–K^+ ATPase; and, (ii) pHi recovery in Na^+-free medium depends on the presence of a chloride current which can be blocked by NPPB and impaired by preincubation in Cl^-–free medium. This finding supports a role for chloride in the function of the H^+ ATPase, which might be electrical shunting or a biochemical interaction.

Key words: MDCK cells — H^+ ATPase — H^+–K^+ ATPase — Intracellular pH — Chloride

Introduction

Madin-Darby canine kidney (MDCK) cells, a permanent cell line derived from dog kidney, are among the best characterized cultured renal epithelial cells (Madin-Darby, 1958). They form monolayers with a characteristic polarized epithelial morphology: brush-border, apical cell-to-cell junctions and lateral spaces (Rabito et al., 1978). MDCK cells display some properties of distal nephron epithelia: hormonal profile with a cAMP response to vasopressin, oxytocin, glucagon, prostaglandin and epinephrine but not to PTH or calcitonin; high activity of Na^+-K^+ ATPase; the expression of a furosemide sensitive $Na^+/K^+/2Cl^-$ symport (Lang & Paulmichl, 1995); and the fact that monoclonal antibodies prepared against MDCK cells bind to the thick ascending limb, distal convoluted tubule, and cortical collecting duct of the dog kidney (Rindler & Saier, Jr., 1981). There are two strains of this cell line, the strain I derived from an early passage (60–70) and the strain II from later passages (100–110) (Barker & Simmons, 1981; Richardson, Scalera & Simmons, 1981). The most distinctive difference between these strains is their electrical resistance. The strain I forms *tight* epithelia with resistance over 3000 ohm · cm^2, whereas the strain II produces *leaky* epithelia with 100 ohm. · cm^2. Valentich (1981) found that low-passage MDCK cultures (strain I) contain two

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Table 1. Solutions

	Solution 1 Control	Solution 2 NH_4Cl	Solution 3 0 Na^+	Solution 4 0 Na^+ Low K^+	Solution 5 0 Na^+ Low K^+ NH_4Cl	Solution 6 0 Cl^-	Solution 7 0 Cl^- 0 Na^+
NaCl	145	125					
KCl	5	5	5	0.5	0.5		
NaH_2PO_4	1	1				1	
Na_2SO_4	1	1				1	
$CaCl_2$	1.8	1.8	1.8	1.8	1.8		
HEPES	30	30	30	30	30	30	30
$MgCl_2$	1	1	1	1	1		
NMDG			147	152	132		147
NH_4Cl		20			20		
Glucose	10	10	10	10	10	10	10
Na^+ gluconate						145	
K^+ gluconate						5	5
Ca^{++} gluconate						1.8	1.8
Mg gluconate						1	1
pH	7.4	8.0	7.4	7.4	7.4	7.4	7.4

Compositions of the solutions are expressed in mM. NMDG, N-methyl-D-glucamine.
NaOH was used in all Na^+ containing solutions to titrate to the appropriate pH, and KOH was used in Na^+ free solution except in low K^+ solution.

cell types, ciliated and nonciliated, and demonstrated their morphological similarities to the principal and intercalated cells of the mammalian collecting tubule. In addition to morphological similarities, there is growing evidence to suggest that MDCK cells—like intercalated cells—are involved both in acid-base regulation and Cl^- transport. The cells possess high carbonic anhydrase activity, bind peanut lectin on their luminal membrane, are involved in channel-mediated rheogenic Cl^- transport, and are the site of both amiloride sensitive Na^+/H^+ and stilbene sensitive Cl^-/HCO_3-exchange (Devuyst et al., 1994; Ebner & Marin-Grez, 1996; Kurtz & Golchini, 1987; Pfaller et al., 1989; Rindler & Saier, 1981).

The vacuolar H^+ ATPase has been shown to be one of the most important agents of acid secretion in the distal nephron (Capasso et al., 1994; Hamm & Hering-Smith, 1993). This is an electrogenic transporter, responsible for a proton current into the renal tubule lumen or into the mucosal compartment of turtle bladder (Steinmetz, 1986). This mechanism of proton secretion depends on the presence of a Cl^- current in the apical membrane of proximal and distal tubule of rat (Fernandez et al., 1997; Zimolo, Montrose & Murer, 1992). Recently, Dominiak and Garg (1994) have shown that MDCK cells have an ATPase activity which is inhibited by N-ethylmaleimide and bafilomycin, a specific inhibitor of the H^+ ATPase.

In the present paper, we discuss the functional activity of the Na^+-independent mechanisms of H^+ secretion in cultured MDCK cells, particularly the proton transport by the vacuolar H^+ ATPase and its chloride dependence.

Materials and Methods

Cell Culture

Wild-type MDCK cells obtained from the American Type Culture Collection (ATCC, Rockville, MD) were used for all experiments (passage 60–70). Serial cultures were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were grown at 37°C, 95% humidified air and 5% carbon dioxide (pH 7.4) in a CO_2 incubator (Lab-Line Instruments, Melrose Park, IL). The cells were harvested with trypsin and then seeded onto sterile glass coverslips, incubated again for 72 hr in the same medium to become confluent.

Fluorescent Measurement of pHi

pHi was monitored using the fluorescent probe 2′,7′-biscarboxyethyl-5,6-carboxyfluorescein (BCECF). Cells grown to confluence on glass coverslips 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 glass coverslips were placed into a thermo-regulated chamber mounted on an inverted epifluorescence microscope (Nikon, TMD). The coverslips were rinsed with the control solution to remove the BCECF containing solution. The measured area under the microscope had a diameter of 260 μm and contained of the order of 42 cells. All experiments were performed at 37°C.

The cells were alternately excited at 440 or 495 nm with a 150 W xenon lamp and the fluorescence emission was monitored at 530 nm by a photomultiplier-based fluorescence system (Georgia Instruments, PMT-4000) at time intervals of 5 sec. The 495/440 excitation ratio corresponds to a specific pHi. At the end of an experiment, calibration of the BCECF signal was achieved by the high K^+—nigericin method

(Thomas et al., 1979). pHi was set approximately equal to pHo by exposing the cells to a solution containing 130 KCl, 20 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 10 μM nigericin, at different values of pH. The 495/440 ratio was measured at different pHo, and the resulting plot of 495/440 ratios *vs.* pH yielded a calibration curve (*see* Fig. 1). The calibration curve was used to convert all fluorescence data to pHi.

To analyze the functional activity of the H^+ transporters we observed the intracellular alkalinization following an acute acid load due to a 20 mM NH_4Cl pulse (solution 2), and calculated 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. Since the rate of pH recovery depends on the value of cell pH achieved by the acid load, we took care to use experiments in which this pH was in the same range, such that these values were not significantly different between the studied groups (*see* Table 2) (Weintraub & Machen, 1989).

Determination of Buffering Capacity

Intracellular buffer capacity (βi) was determined using the technique described by Boyarsky (1988) and calculated precisely as described by Weintraub (1989). βi refers to the ability of intrinsic cellular components excluding HCO_3^-/CO_2 to buffer changes in pHi, and thus estimations of βi were carried out in HEPES—buffered solutions. βi is defined as Δ[base]/ΔpHi and is most precisely estimated in cells in which pHi regulatory mechanisms have been blocked. H^+–HCO_3^- membrane transporters were blocked by a 0 mM Na^+, 0.5 mM K^+ solution plus 4.6 × 10^{-8} M concanamycin, and then the external [NH_4^+] was stepwise lowered from 20 to 0 mM in the nominal absence of HCO_3^-/CO_2 (solution 5). Different concentrations of NH_4Cl were obtained by mixing solutions 4 and 5. The rate of transmembrane H^+ flux (J_{H+}) was calculated from the following equation: J_{H^+} = (dpHi/dt) × βi, where dpHi/dt is the initial rate of pHi recovery and βi is the averaged cytosolic buffering capacity.

Solutions and Reagents

The composition of the solutions utilized in this work is described in Table 1. These solutions had an osmolality between 325 and 330 mOsm, which is the value found in the culture medium used for these cells. This osmolality was used to avoid changes when the cells were transferred from the culture medium to the experimental solutions. Concanamycin A was obtained from Mikrobielle Chemie, Ciba, Basel, Switzerland; NPPB (5-Nitro–2-(3-phenylpropylamino–benzoic acid) from Prof. R. Greger, Freiburg University, Germany. Nigericin from Sigma, and Schering 28080 from Schering-Plough (Liberty Corner, NJ). BCECF-AM was from Molecular Probes, Junction City, OR. All other applied chemicals were of analytical grade and obtained from Sigma.

Statistics

The data are presented as mean values ± SEM. Statistical comparisons were made by analysis of variance followed by the Student-Newman-Keuls contrast test. Differences were considered significant if $P < 0.05$.

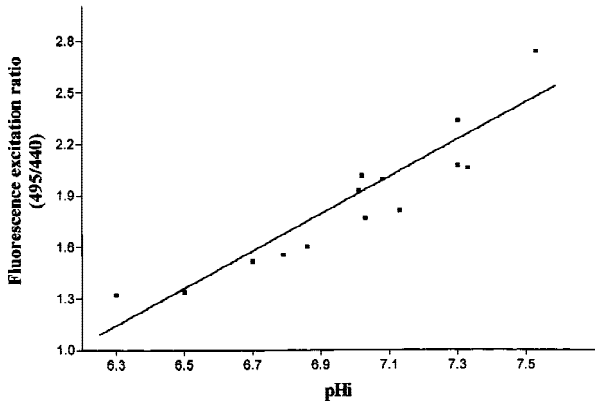

Fig. 1. Calibration of BCECF fluorescence ratio in MDCK cells. Monolayers of MDCK cells were loaded with 12 μM BCECF-AM, mounted on the thermoregulated chamber and the fluorescence excitation ratio was measured as pHo was varied by the high K^+–nigericin technique (*see* Materials and Methods).

Results

Cells were first bathed with Na^+ solution (solution 1, Table 1); they showed an average resting pHi of 7.17 ± 0.01 (n = 23). The role of the H^+ transport mechanisms in cell pH recovery was examined following the acidification of pHi with the NH_4Cl pulse technique. After 2-min exposure to 20 mM NH_4Cl, during which cell pH increased transiently, NH_4Cl removal caused a rapid acidification of pHi as a result of NH_3 efflux (Fig. 2*A*). In the presence of external 145 mM Na^+ the pHi recovery rate (initial 2 min) was 0.121 ± 0.006 pH units/min (n = 23), and the final pHi was not significantly different from the basal value: 7.11 ± 0.02 *vs.* 7.17 ± 0.01. This pHi recovery rate was markedly decreased after removal of Na^+ (solution 3, Table 1), to 0.069 ± 0.004 (n = 16) (Figs. 2*B* and 3), and in this group recovery was not complete, 6.77 ± 0.06 *vs.* 7.14 ± 0.02. The recovery of pHi observed in these conditions (Na^+ free) indicates the existence of Na^+ independent proton extrusion pathways, probably proton ATPases.

To investigate if Na^+ independent pHi recovery was due to the activity of a H^+ ATPase, experiments on the effects of concanamycin on this recovery were performed. This agent is reported to have a larger specificity for V type ATPases than bafilomycin (Drose et al., 1993). The pHi recovery after the NH_4Cl pulse was reduced to 0.042 ± 0.005 (n = 12) when concanamycin 4,6 × 10^{-8} M was added to the zero Na^+ solution. When using a solution with zero Na^+, low K^+ (0.5 mM) plus concanamycin (solution 4), pHi recovery fell further to 0.023 ± 0.006 (n = 14), significantly less than with the previous solution (Figs. 2*D* and 3). This result is compatible with the presence of an H^+–K^+ ATPase in these cells, responsible with the vacuolar H^+ ATPase for the

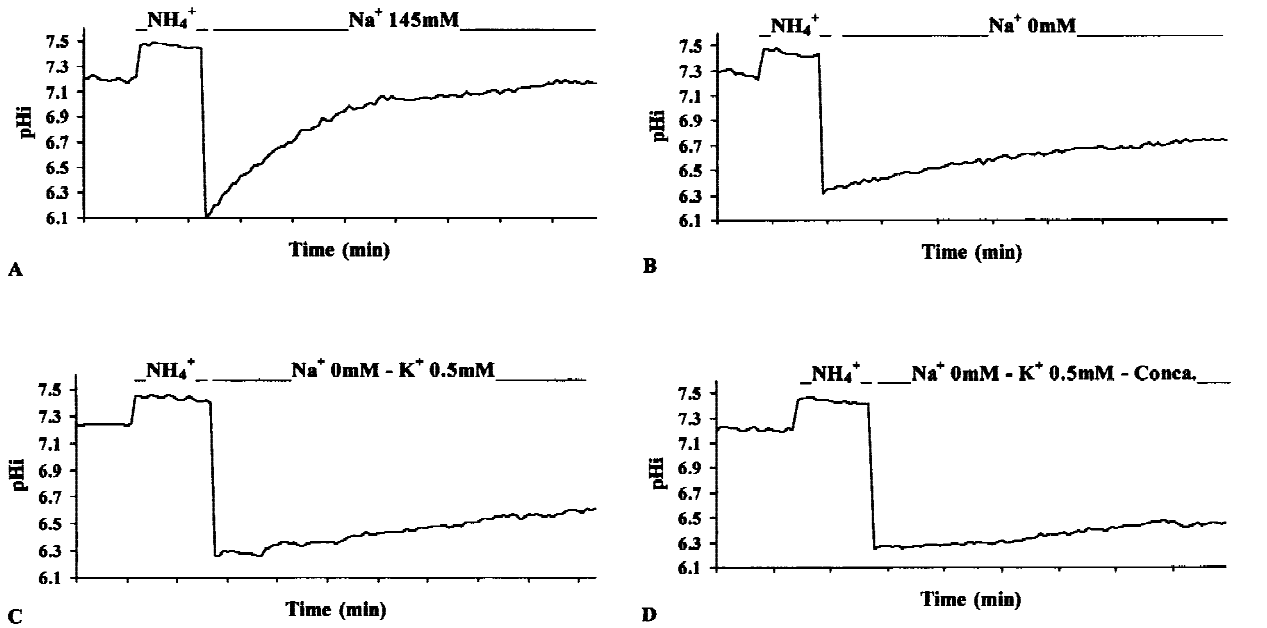

Fig. 2. Acidification of pHi following the removal of NH_4Cl^-. Cells were bathed in a solution containing 20 mM NH_4Cl^- for 2 min. Following the removal of NH_4Cl^-, pHi decreases acutely due to rapid efflux of NH_3. The figure shows four representative experiments. (*A*) The initial fall in pHi is followed by a recovery of pHi towards the basal value in the presence of 145 mM extracellular Na^+. (*B*) Removal of extracellular Na^+ resulted in a significant inhibition of the pHi recovery. (*C*) Low K^+ (0.5 mM) solution caused a significant reduction in the Na^+-independent pHi recovery. (*D*) The pHi recovery observed in the previous experiment was further inhibited by 4.6×10^{-8} M concanamycin.

Na^+ independent pHi recovery. To further examine this point we added 5×10^{-5} M Schering 28080 (a specific inhibitor of the H^+–K^+ ATPase) to the zero Na^+ solution, and the pHi recovery was also significantly reduced (Table 2 and Fig. 3). This rate was not different from the zero Na^+, low K^+ group, 0.049 ± 0.006 ($n = 11$) *vs.* 0.047 ± 0.005 ($n = 14$).

These data show that the wild type of MDCK cells possess two Na^+-independent mechanisms of proton extrusion, a H^+ ATPase and a H^+–K^+ ATPase, like the intercalated cells of the distal nephron.

Role of Cl^- Channels in Na^+-Independent Proton Secretion

It has been shown that in several cell types the elimination of Cl^- from the medium or the use of Cl^- channel blockers leads to impairment of the acidification caused by H^+ ATPase (Kaunitz, Gunther & Sachs, 1985; Vieira, Slotki & Cabantchik, 1995). Previous studies of our laboratory showed that in the cortical distal tubule of rat kidney blocking of Cl^- channels by NPPB inhibits H^+ secretion (Fernandez et al., 1997). Figure 4 shows that Na^+-independent pHi recovery was significantly reduced from 0.069 ± 0.004 to 0.042 ± 0.004 ($n = 14$) when 10^{-5} M NPPB (a specific inhibitor of Cl^- channels in renal tubules) was utilized. When a Na^+- and Cl^--free medium was introduced after the NH_4Cl pulse the rate of pH recovery was not different from that observed in a 0 Na^+ solution (0.073 ± 0.007, $n = 11$, *vs.* 0.069 ± 0.004). However, when the cells were preincubated for 8 min in 0 Cl^-/normal Na^+ (solution 6) before the ammonium pulse (also without Cl^-), pHi recovery fell to 0.041 ± 0.007, $n = 12$, significantly lower than with the 0 Na^+ solution. Considering that Ca^{++} activity in gluconate solutions is lower than in chloride Ringer, we increased Ca^{++} to 7 mM in an additional experimental group. In this solution, dpH/dt was 0.041 ± 0.006 ($n = 5$), not different from the Ca^{++} 1.8 mM group, indicating that the reduction in dpH/dt was not due to the low Ca^{++} activity. When using the zero Na^+, low K^+ solution plus NPPB this pHi recovery fell further to 0.030 ± 0.004 ($n = 14$) (Table 2 and Fig. 4). These results support the important role of Cl^- channels for the function of H^+ ATPase that we found in the in vivo distal tubule.

Recently it has been shown that some epithelial chloride channels are sensitive to inhibition at acidic intracellular pH values (Guinamard, Paulais & Teulon, 1996). Since many of the chloride channel blockers currently in use are weak acids, it is possible that part of the inhibitory action of these agents may result from their effects upon pHi rather than from specific interaction with the channel. To study this aspect we added 10^{-5} M NPPB to the control solution and observed the effect on the basal pHi. The addition of the chloride blocker did not modify the basal pHi of the monolayers significantly,

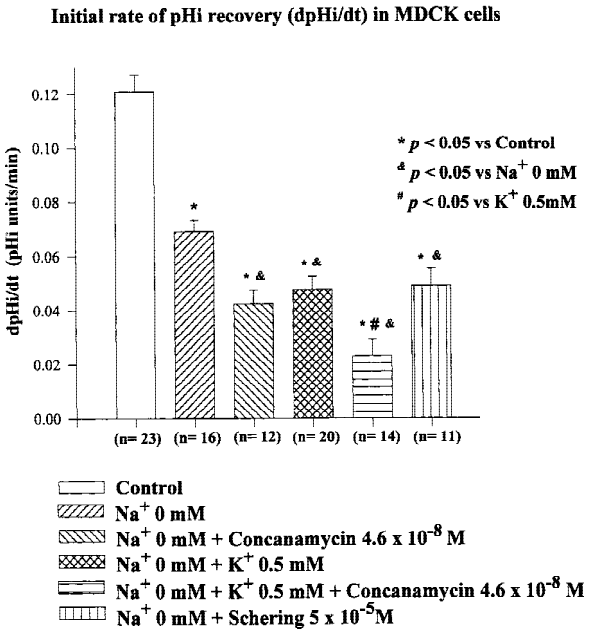

Fig. 3. Initial rate of pHi recovery following acute intracellular acidification in MDCK cells. Na^+-independent pHi recovery was significantly inhibited by 4.6×10^{-8} M concanamycin, a low K^+ solution (0.5 mM) and 5×10^5 Schering 28080. No. of experiments in parentheses.

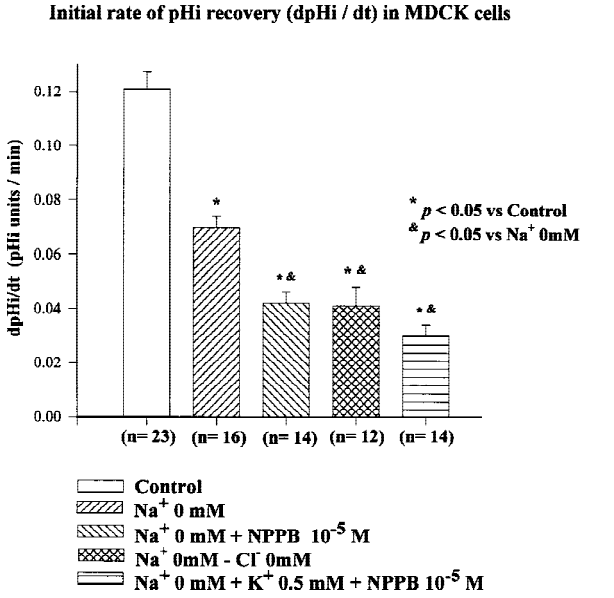

Fig. 4. Initial rate of pHi recovery following acute intracellular acidification in MDCK cells. Na^+-independent pHi recovery was significantly inhibited by 10^{-5} M NPPB and by intracellular chloride depletion. No. of experiments in parentheses.

7.15 ± 0.04 after NPPB *vs.* 7.19 ± 0.02 ($n = 7$) before the drug.

Intracellular Buffering Capacity and Proton Flux

To allow for the calculation of proton fluxes at different pHi values the intracellular buffering capacity (βi) was determined at different pHi as described in Materials and Methods. Buffer capacity depends significantly on pHi over the tested pHi range of 5.8–7.2. It increases exponentially as the pHi is lowered.

The rate of transmembrane H^+ flux (J_{H^+}) was calculated from the following equation: $J_{H+} = dpHi/dt \times \beta i$, where dpHi/dt is the rate of pHi recovery after the acid load and βi is the averaged cytosolic buffering capacity. Because most pH regulatory mechanisms are sensitive to pHi, care must be taken to compare rates at the same pHi value. We compared the rates of H^+ extrusion at pHi 6.65, and this is shown in Fig. 5. In our experiments the J_{H+} of the Na^+-independent mechanisms of pHi recovery was larger than that of the Na^+ dependent: 9.73 mM/min *vs.* 7.42 mM/min.

Table 2. Summary of pH_i responses in MDCK cells to addition of different solutions after an acute acid load

	n	pH_i Initial	pH_i Acid Load	ΔpH_i	ΔpH_i/min
Control	23	7.17 ± 0.01	6.57 ± 0.06	0.51 ± 0.05	0.121 ± 0.006
Na^+ 0 mM	16	7.14 ± 0.02	6.45 ± 0.07	0.34 ± 0.04[a]	0.069 ± 0.004
Na^+ 0 mM + Concanamycin	12	7.17 ± 0.02	6.58 ± 0.07	0.15 ± 0.02[a,b]	0.042 ± 0.005[c]
Na^+ 0 mM K^+ 0.5 mM	20	7.17 ± 0.01	6.52 ± 0.04	0.23 ± 0.02[a,b]	0.047 ± 0.005[c]
Na^+ 0 mM K^+ 0.5 mM + Concanamycin	14	7.17 ± 0.01	6.61 ± 0.06	0.10 ± 0.01[a,b]	0.023 ± 0.006[c,d]
Na^+ 0 mM + Sch 28080	11	7.18 ± 0.01	6.48 ± 0.06	0.18 ± 0.02[a,b]	0.049 ± 0.006[c]
Na^+ 0 mM + NPPB	14	7.20 ± 0.01	6.58 ± 0.05	0.17 ± 0.02[a,b]	0.042 ± 0.004[c]
Na^+ 0 mM + Cl^- 0 mM	12	7.17 ± 0.01	6.66 ± 0.06	0.31 ± 0.04[a]	0.041 ± 0.007[c]
Na^+ 0 mM K^+ 0.5 mM + NPPB	14	7.16 ± 0.01	6.67 ± 0.04	0.13 ± 0.01[a,b]	0.030 ± 0.004[c]

Values are means ± SE; *n*, no. of observations.
ΔpH_i (pH_i recovery–pH_i acid load). ΔpH_i/min, pH_i recovery rate.
Concanamycin 4.6×10^{-8} M, NPPB 10^{-5} M, Schering 28080 5×10^{-5} M.
[a] $P < 0.05$ vs. control ΔpH_i; [b] $P < 0.05$ vs. Na^+ 0 mM ΔpH_i; [c] $P < 0.05$ vs. Na^+ 0 mM ΔpH_i/min; [d] $P < 0.05$ vs. Na^+ 0 mM − K^+ 0 mM ΔpH_i/min.

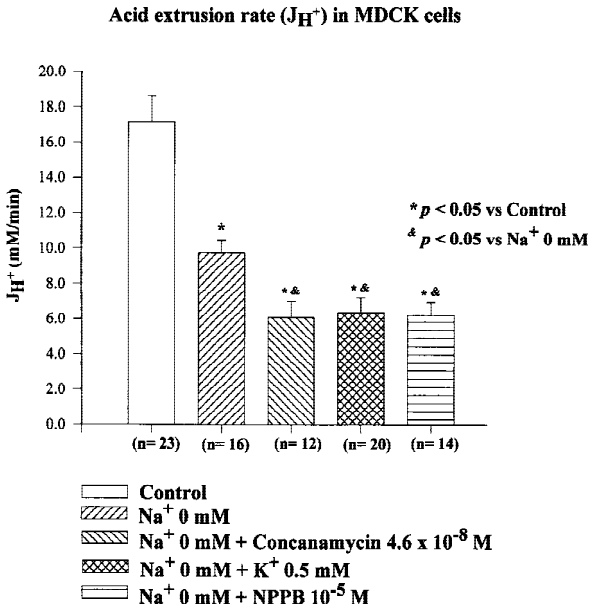

Fig. 5. Acid extrusion rate (J_H^+) in MDCK cells following a NH_4Cl pulse. J_H^+ was obtained from the product of dpHi/dt and intracellular buffer capacity (βi) at a pHi of 6.65. No. of experiments in parentheses.

Discussion

If grown to confluence on impermeable culture dishes, MDCK cells display net ion and fluid transport from the apical to the basolateral cell side, leading to the formation of domes, fluid filled blisters between epithelial cell layers and culture dishes; a process stimulated by aldosterone (Oberleithner, Vogel & Kersting, 1990*a;* Rabito et al., 1978). This is the result of net transport of NaCl, which is also a property of the principal cells of the collecting duct. On the other hand, MDCK cells are capable of secreting H^+ and Cl^- (Brown & Simmons, 1981; Oberleithner et al., 1990*b*), and present the activity of a Cl^-/HCO_3^- exchanger (Ebner et al., 1996; Kurtz et al., 1987). In addition to these characteristics, the MDCK cells share the following properties with intercalated cells: carbonic anhydrase activity (Pfaller et al., 1989), peanut lectin binding (Kersting et al., 1990), Na^+/H^+ exchange (Rindler & Saier, 1981), and presence of H^+ ATPases (Dominiak & Garg, 1994; Feifel et al., 1997).

From the morphological point of view, there are many similiarites between the MDCK monolayer and the mammalian collecting duct. Valentich (1981), working with low passage MDCK cultures, i.e., passage 60 to 65 (similar to our experiments), identified two cell types by electron microscopy. One type was ciliated, with light cytoplasm and few apical vesicles; and the other was nonciliated, with dark cytoplasm and apical vesicles. The ciliated cells showed similarities with the principal cells, and the nonciliated cells correspond to the intercalated cells of the collecting duct, including the presence of carbonic anhydrase activity (Nakazato, Suzuki & Saruta, 1989). The majority of these studies utilized MDCK cells grown on collagen gels. When the cells were grown to confluence on plastic or glass substrate the morphological heterogeneity was attenuated but did not disappear (Valentich, 1981).

Devuyst et al. (1994), working with three immunocytochemical markers of intercalated cells, (peanut lectin binding capacity, PNA, carbonic anhydrase activity and antibodies against human erythrocyte band 3 protein) documented the heterogeneity of the MDCK cell line (low passage). About 30% of the MDCK cells bind PNA, two-thirds of them are also positive for carbonic anhydrase, and 5% of the cells display band 3 immunoreactivity. These immunoreactive cells may correspond to intercalated cells, and would be the cells where the proton ATPases described in the present work are localized. This heterogeneity of the strain I of MDCK cells was confirmed by Gekle et al. (1994), who cloned two MDCK cell subtypes denominated as C7 and C11. The C11 clone establishes a transepithelial Cl^- and pH gradient, and two-thirds of these cells exhibited a detectable PNA binding, and thus resemble intercalated cells of the renal collecting duct.

In the present study the MDCK cells were from passage 60 to 70, thus from cell strain I according to the classification of Richardson et al. (1981). We did not distinguish between the two cell types present in this preparation.

The present work discloses the different mechanisms of pHi recovery in the MDCK cells after an acid load, including the Na^+/H^+ exchanger, the H^+–K^+ ATPase and the vacuolar H^+ ATPase. The more important of these is the Na^+/H^+ exchanger, since the removal of extracellular Na^+ led to a 40% reduction in the rate of pHi recovery (Fig. 3). The presence of this component of proton secretion is known since Rindler's work (1981), who showed the relation between Na^+ transport and pHi changes, and its sensitivity to amiloride. Other authors confirmed his results, and discussed the kinetic properties of this transport (Goldfarb & Nord, 1987; Selvaggio et al., 1986). Several studies on the polarity of the Na^+/H^+ transporter are available but no consensus was obtained. Most studies localized the exchanger to the basolateral membrane (Rosenberg et al., 1991; Vilella et al., 1992), but evidence for apical localization was also reported (Oberleithner et al., 1990*b*). Recently, it was shown that the isoform NHE1 of the Na^+/H^+ exchanger (the only isoform expressed spontaneously in these cells) was expressed at both sides of the polarized MDCK cells, with a preference for the apical side (70%) (Noel, Roux & Pouysségur, 1996). In our studies, performed on impermeant glass supports, it was not possible to define the polarity of the studied transporters.

We were able to show Na^+-independent pHi recov-

ery after an acute acid load (Fig. 2*B*) in our preparation. Considering that the epithelium utilized in our study has cells with characteristics of intercalated cells, it is probable that H^+ ion transporting ATPases were responsible for the Na^+ independent pHi recovery. The reduction of the extracellular K^+ to 0.5 mM or the addition of 5×10^{-5} M Schering 28080 caused a significant reduction of the Na^+-independent pHi recovery (Fig. 3), confirming the presence of a H^+–K^+ ATPase in the membrane of the MDCK cells as had been shown before (Oberleithner et al., 1990*c;* Feifel et al., 1997). When using a low K^+ solution plus concanamycin the Na^+ independent pHi recovery was almost completely inhibited (Figs. 2*D* and 3), showing the presence of a vacuolar H^+ ATPase. This functional evidence agrees with the biochemical results that have shown an ATPase activity which is inhibited by N–ethylmaleimide and bafilomycin (Dominiak et al., 1994).

In summary, the monolayers of MDCK cells utilized in the present study have two Na^+ independent proton secretion mechanisms: a vacuolar H^+ ATPase and a H^+–K^+ ATPase, mechanisms similar to those found in the intercalated cells of mammalian collecting duct.

Role of Cl^- Channels in H^+ Secretion by H^+ ATPase

It has been reported that not only the electrogenicity of H^+ ATPase is affected by the presence of Cl^- ions, but also that H^+ transport by this transporter depends on this anion (Gluck, 1993). When the activity of H^+ ATPase was measured by microfluorometry at the single cell level in rat proximal tubules loaded with BCECF, H^+ extrusion was reduced by lowering intracellular Cl^- (Zimolo et al., 1992). Recently, we observed a significant reduction of $J_{HCO_3^-}$ in the late cortical distal tubule of the rat when apical chloride channels were blocked with NPPB. These data demonstrate that H^+ secretion by this segment also depends on an apical chloride current.

Transepithelial chloride secretion is observed in confluent MDCK cells, stimulated by epinephrine, ATP or prostaglandin (Simmons, 1981; Simmons, 1991). This chloride secretion is accomplished by a chloride channel in the apical cell membrane, an anion channel of 460pS which is activated by sudden changes of the potential difference across the patch (Kolb, Brown & Murer, 1985). This apical Cl^- current is inhibited by NPPB, a specific chloride channel blocker in different nephron segments (Wangemann et al., 1986). A chloride channel was also identified in these cells by an expression-cloning strategy (Paulmichl et al., 1992). The isolation of a rat cDNA for the MDCK type chloride channel (RKCl) which is expressed in many tissues including the kidney has been reported (Ishibashi, Rector, Jr. & Berry, 1993). *In situ* hybridization showed that RKCl mRNA is expressed in the renal cortex and medulla (Abe et al., 1996). Recently, it was demonstrated that CFTR (cystic fibrosis transmembrane conductance regulator) is expressed constitutively in MDCK strain I, that this anion conductance is stimulated by cAMP, is sensitive to NPPB, and is localized in the apical plasma membrane (Mohamed et al., 1997).

These findings led us to study the chloride dependence of Na^+ independent pH recovery in MDCK cells. This pHi recovery was significantly inhibited when NPPB was added to the zero Na^+ solution (Fig. 4). This effect appears to be linked to proton secretion by the vacuolar H^+ ATPase, since when NPPB is added to cells incubated in zero Na^+, low K^+ solution, a situation where the H^+–K^+ ATPase is inhibited, a further impairment of the rate of pHi recovery was observed (Fig. 4). Unspecific effects of NPPB on several H^+ ion transport parameters have been reported. At NPPB concentrations of 25 μM and up, a significant protonophore effect occurs, and the rate of transmembrane proton flux increases in human neutrophils (Luckacs et al., 1991). In addition, 100 μM NPPB decreases neutrophil cell ATP to 65% of controls. These effects have been shown to increase cell pH in high K medium and to decrease cell pH with low K (NMDG) medium. In our experiments, we used a NPPB concentration (10 μM) at which such alterations have not been observed. In addition, we have not found significant alterations of basal MDCK cell pH when these cultures were incubated with 10^{-5} M NPPB. When MDCK cells are incubated in a Cl^--free medium, the Na^+-independent H^+ extrusion is also impaired (Fig. 4). The finding that acute extracellular superfusion with Cl^--free solutions without preincubation does not affect H^+ transport indicates that it is probably a reduction of intracellular Cl^- that determines impairment of the relevant Cl^- current, as shown before for proximal renal tubule and thick ascending limb (Zimolo et al., 1992; Greger, 1985). These data demonstrate that the Na^+-independent proton secretion by MDCK cells depends on a chloride current, the inhibition of which reduces the activity of the vacuolar H^+ ATPase.

The chloride-dependence of H^+ ion secretion in MDCK cells may reflect apical secretion of Cl^- compensating the transfer of positive charge by the H^+ ATPase. However, it may also be the consequence of stimulation of proton pump activity by Cl^- as proposed for rat renal medullary microsomal vesicles (Kaunitz et al., 1985).

In conclusion, the results of the present study demonstrate that:

i. MDCK cells, strain I, have two Na^+-independent mechanisms of pHi recovery, a concanamycin-sensitive H^+ ATPase and a K^+ dependent, Schering 28080-sensitive H^+–K^+ ATPase.
ii. pHi recovery in Na^+-free medium depends on the presence of a chloride current which can be blocked

by NPPB and impaired by preincubation in Cl^--free medium. This finding supports a role for chloride in the function of the H^+ ATPase, which might be electrical shunting or a biochemical interaction.

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References

Abe, T., Takeuchi, K., Takahashi, N., Taniyama, Y., Tsutsumi, E., Abe, K. 1996. Intrarenal mRNA expression of the rat MDCK-type chloride channel. *Nephron* **73:**23–26

Barker, G., Simmons, N.L. 1981. Identification of two strains of cultured canine renal epithelial cells (MDCK cells) which display entirely different physiological properties. *Q. J. Expt. Physiol.* **66:**61–72

Boyarsky, G., Ganz, M.B., Sterzel, R.B., Boron, W.F. 1988. pH regulation in single glomerular mesangial cells. I. Acid extrusion in absence and presence of HCO3-. *Am. J. Physiol.* **255:**C844–C856

Brown, C.A., Simmons, N.L. 1981. Catecholamine stimulation of Cl^- secretion in MDCK cell epithelium. *Biochim. Biophys. Acta* **649:**427–435

Capasso, G., Malnic, G., Wang, T., Giebisch, G. 1994. Acidification in mammalian cortical distal tubule. *Kidney Int.* **45:**1543–1554

Devuyst, O., Beauwens, R., Denef, J.F., Crabbe, J., Abramow, M. 1994. Subtypes of Madin-Darby canine kidney (MDCK) cells defined by immunocytochemistry: further evidence for properties of renal collecting duct cells. *Cell Tissue Res.* **277:**231–237

Dominiak, D.M., Garg, L.C. 1994. H^+ ATPase in Madin-Darby canine kidney (MDCK) cells, regulation by carbachol. *FASEB J.* **8:**A300

Drose, S., Bindseil, K.U., Bowman, E.J., Siebers, A., Zeeck, A., Altendorf, K. 1993. Inhibitory effect of modified Bafilomycins and Concamycins on P- and V-Type Adenosinetriphosphatases. *Biochemistry* **32:**3902–3906

Ebner, S., Marin-Grez, M. 1996. Video-imaging micro-fluorometric assessment of luminal chloride/bicarbonate exchange activity in Madin-Darby canine kidney cells: Influence of cell density, 4,4′-diisothiocyano-2,2′-disulfonic stilbene and acetazolamide. *Biol. Chem. Hoppe Seyler* **377(5):**275–281

Feifel, E., Krall, M., Geibel, J., Pfaller, W. 1997. Differential activities of H^+ extrusion systems in MDCK cells due to extracellular osmolality and pH. *Am. J. Physiol.* **273:**F499–F506

Fernández, R., Bosqueiro, J.R., Cassola, A.C., Malnic, G. 1997. Role of Cl^- in electrogenic H^+ secretion by cortical distal tubule. *J. Membr. Biol.* **157(2):**193–201

Gekle, M., Wünsch, S., Oberleithner, H., Silbernagl, S. 1994. Characterization of two MDCK cell subtypes as a model system to study principal cell and intercalated cell properties. *Pfluegers Arch.* **428:**157–162

Gluck, S.L. 1993. The vacuolar H^+ ATPases: Versatile proton pumps participating in constitutive and specialized functions of eukaryotic cells. *International Review of Cytology* **137C:**105–137

Goldfarb, D., Nord, E.P. 1987. Asymmetric affinity of Na^+-H^+ antiporter for Na^+ at the cytoplasmic versus external transport site. *Am. J. Physiol.* **253:**F959–F968

Greger, R. 1985. Ion transport mechanisms in thick ascending limb of Henle's loop of mammalian nephron. *Physiol. Rev.* **65:**760–797

Guinamard, R., Paulais, M., Teulon, J. 1996. Inhibition of a small-conductance cAMP-dependent Cl^- channel in the mouse thick ascending limb at low internal pH. *J. Physiol.* **490:**759–765

Hamm, L.L., Hering-Smith, K.S. 1993. Acid-base transport in the collecting duct. *Sem. Nephrol.* **13:**246–255

Ishibashi, K., Rector, F.C., Jr., Berry, C.A. 1993. Role of Na-dependent Cl/HCO_3 exchange in basolateral Cl transport of rabbit proximal tubules. *Am. J. Physiol.* **264:**F251–F258

Kaunitz, J.D., Gunther, R.D., Sachs, G. 1985. Characterization of an electrogenic ATP and chloride-dependent proton translocating pump from Rat Renal Medulla. *J. Biol. Chemistry* **260:**11567–11573

Kersting, U., Schwab, A., Treidtel, M., Pfaller, W., Gstraunthaler, G., Steigner, W., Oberleithner, H. 1990. Differentiation of MDCK cells: structure–function relationship using lectin binding. *Kidney Int.* **37:**1163

Kolb, H.A., Brown, C.D.A., Murer, H. 1985. Identification of a voltage-dependent anion channel in the apical membrane of a Cl^- secretory epithelium (MDCK). *Pfluegers Arch.* **403:**262–265

Kurtz, I., Golchini, K. 1987. Na^+-independent Cl^--HCO_3^- exchange in Madin-Darby canine kidney cells: role in intracellular pH regulation. *J. Biol. Chem.* **262:**4516–4520

Lang, F., Paulmichl, M. 1995. Properties and regulation of ion channels in MDCK cells. *Kidney Int.* **48:**1200–1205

Luckacs, G.L., Nanda, A., Rotstein, O.D., Grinstein, S. 1991. The chloride channel blocker 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB) uncouples mitochondria and increases the proton permeability of the plasma membrane in phagocytic cells. *FEBS Lett.* **288:**17–20

Madin, S.H., Darby, N.B. 1958. As catalogued in American Type Culture Collection. *Catalog of Strains* **2:**574–576

Mohamed, A., Ferguson, D., Seibert, F.S., Cai, H., Kartner, N., Grinstein, S., Riordan, J.R., Lukacs, G. 1997. Functional expression and apical localization of the cystic fibrosis transmembrane conductance regulator in MDCK I cells. *Biochem. J.* **322:**259–265

Nakazato, Y., Suzuki, H., Saruta, T. 1989. Characterization of subclones of Madin-Darby canine kidney renal epithelial cell line. *Biochim. Biophys. Acta* **1014:**57–65

Noel, J., Roux, D., Pouysségur, J. 1996. Differential localization of Na^+/H^+ exchanger isoforms (NHE1 and NHE3) in polarized epithelial cell lines. *Journal of Cell Science* **109:**929–939

Oberleithner, H., Steigner, W., Silbernagl, S., Vogel, U., Gstraunthaler, G., Pfaller, W. 1990*c*. Madin-Darby canine kidney cells. III. Aldosterone stimulates an apical H^+/K^+ pump. *Pfluegers Arch.* **416:**540–547

Oberleithner, H., Vogel, U., Kersting, U. 1990*a*. Madin-Darby canine kidney cells. I. Aldosterone-induced domes and their evaluation as a model system. *Pfluegers Arch.* **416:**526–532

Oberleithner, H., Vogel, U., Kersting, U., Steigner, W. 1990*b*. Madin-Darby canine kidney cells. II. Aldosterone stimulates Na^+/K^+ and Cl^-/HCO_3^- exchange. *Pfluegers Arch.* **416:**533–539

Paulmichl, M., Li, Y., Wickman, K., Ackerman, M.J., Peralta, E., Clapham, D. 1992. New mammalian chloride channel identified by expression cloning. *Nature* **356:**238–241

Pfaller, W., Gastraunthaler, G., Kersting, U., Oberleithner, H. 1989. Carbonic anhydrase activity in Madin Darby canine kidney cells. *Renal Physiol. Biochem.* **12:**328–337

Rabito, C.A., Tchao, R., Valentich, J., Leighton, J. 1978. Distribution and characteristics of the occluding junctions in a monolayer of a cell line (MDCK) derived from canine kidney. *J. Membrane Biol.* **43:**351–365

Richardson, J., Scalera, V., Simmons, N.L. 1981. Identification of two strains of MDCK cells which resemble separate nephron tubule segments. *Biochim. Biophys. Acta* **673:**26–36

Rindler, M.J., Saier, M.H., Jr. 1981. Evidence for Na^+/H^+ antiport in

cultured dog kidney cells (MDCK). *J. Biol. Chem.* **256:**10820–10825

Rosenberg, S.O., Berkowitz, P.A., Li, L., Schuster, V.L. 1991. Imaging of filter-grown epithelial cells: MDCK Na^+-H^+ exchanger is basolateral. *Am. J. Physiol.* **260:**C868–C876

Selvaggio, A.M., Schwartz, J., Bengele, H.H., Alexander, E.A. 1986. Kinetics of the Na^+-H^+ antiporter as assessed by the change in intracellular pH in MDCK cells. *Am. J. Physiol.* **251:**C558–C562

Simmons, N.L. 1981. Stimulation of Cl^- secretion by exogenous ATP in cultured MDCK epithelial monolayers. *Biochim. Biophys. Acta* **646:**231–242

Simmons, N.L. 1991. Chloride secretion stimulated by prostaglandin E1 and by forskolin in a Canine Renal epithelial cell line. *J. Physiol.* **432:**459–472

Steinmetz, P.R. 1986. Cellular organization of urinary acidification. *Am. J. Physiol.* **251:**F173–F187

Thomas, J., Buchsbaum, R., Zimniak, A., Racker, E. 1979. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry* **18:**2210–2218

Valentich, J. 1981. Morphological similarities between the dog kidney cell line MDCK and the mammalian cortical collecting tubule. *Ann. N.Y. Acad. Sci.* **372:**384–405

Vieira, L., Slotki, I., Cabantchik, Z.I. 1995. Chloride conductive pathways which support electrogenic H^+ pumping by Leishmania major promastigotes. *J. Biol. Chem.* **270:**5299–5304

Vilella, S., Guerra, L., Helmle-Kolb, C., Murer, H. 1992. Characterization of basolateral Na/H exchange (Na/H-1) in MDCK cells. *Pfluegers Arch.* **420:**275–281

Wangemann, P., Wittner, M., DiStefano, A., Englert, H.C., Lang, H.J., Schlatter, E., Greger, R. 1986. Cl^- channel blockers in the thick ascending limb of the loop of Henle. Structure activity relationship. *Pfluegers Arch.* **407:**S128–S141

Weintraub, W.H., Machen, T.E. 1989. pH regulation in hepatoma cells: roles for Na-H exchange, Cl-HCO_3 exchange, and Na-HCO_3 cotransport. *Am. J. Physiol.* **257:**G317–G327

Zimolo, Z., Montrose, M.H., Murer, H. 1992. H^+ extrusion by an apical vacuolar-type H^+-ATPase in rat renal proximal tubules. *J. Membrane Biol.* **126:**19–26