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Fused Cells of Frog Proximal Tubule: II. Voltage-Dependent Intracellular pH

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Summary. Experiments were performed in intact proximal tubules of the doubly perfused kidney and in fused proximal tubule cells of *Rana esculenta* to evaluate the dependence of intracellular pH (pH_i) on cell membrane potential applying pH-sensitive and conventional microelectrodes. In proximal tubules an increase of the K^+ concentration in the peritubular perfusate from 3 to 15 mmol/liter decreased the peritubular cell membrane potential from -55 ± 2 to -38 ± 1 mV paralleled by an increase of pH_i from 7.54 ± 0.02 to 7.66 ± 0.02. The stilbene derivative DIDS hyperpolarized the cell membrane potential from -57 ± 2 to -71 ± 4 mV and led to a significant increase of the K^+-induced cell membrane depolarization, but prevented the K^+-induced intracellular alkalinization. Fused proximal tubule cells were impaled by three microelectrodes simultaneously and cell voltage was clamped stepwise while pH_i changes were monitored. Cell membrane hyperpolarization acidified the cell cytoplasm in a linear relationship. This voltage-induced intracellular acidification was reduced to about one-third when HCO_3 ions were omitted from the extracellular medium. We conclude that in proximal tubule cells pH_i depends on cell voltage due to the rheogenicity of the HCO_3^- transport system.

Key Words proximal tubule · cell fusion · intracellular pH · voltage clamp · frog kidney

Introduction

Renal regulation of plasma bicarbonate concentration plays a central role in the control of systemic pH homeostasis. In cytoplasm of proximal tubule cells H^+ and HCO_3^- ions are being formed from the carbonic anhydrase catalyzed hydration of CO_2. The amiloride-inhibitable Na^+/H^+ exchanger of the luminal cell membrane extrudes H^+ into the tubule lumen while HCO_3^- leaves the cell via a conductive pathway or, more specifically, via a rheogenic Na^+/HCO_3^- cotransporter [6, 7, 10, 12, 13, 19, 21]. Recently we have found that the cell membrane potential plays an important role in the control of H^+ secretion in frog diluting segment [24, 25]. Depolarization of the cell membrane potential reduces H^+ secretion while hyperpolarization stimulates transepithelial H^+-secretory flux. The signal transducer between cell voltage and the Na^+/H^+ exchanger is most likely intracellular pH (pH_i), because on the one hand, cytoplasmic H^+ activity can be altered by cell membrane potential and, on the other hand, the Na^+/H^+ exchanger itself is sensitive to pH_i [3]. Therefore, we postulate that due to the rheogenicity of the basolateral HCO_3^- transport system, the cell membrane potential could determine net transfer of HCO_3^- and thus alter pH_i. We tested this hypothesis in intact proximal tubules and fused proximal tubule cells of the amphibian kidney. In the intact tubules the cell membrane potential was depolarized by various K^+ concentrations in the peritubular perfusate. In fused proximal tubule cells the membrane potential was hyperpolarized stepwise applying voltage-clamp techniques. The results show that there is indeed a strong linear relationship between pH_i and cell membrane potential which could play a crucial role in the maintenance of H^+ homeostasis.

Materials and Methods

I. Experiments in Intact Tubules

Kidney Preparation and Perfusion

Preparation and perfusion methods of the frog kidney were described in detail previously [19, 27]. Briefly, *Rana esculenta* (Fa. Stein, Bad Lauingen, FRG) were decapitated and pithed, the abdomen opened, the stem of the coeliac mesenteric artery ligated; stomach and intestine were removed. The kidneys were isolated and placed on a plexiglass dish chamber. The *dorsal* surface of the kidney was exposed for micropuncture. The aorta was cannulated by a polyethylene catheter advanced to the point of union with the arteria iliaca communis. Another catheter was placed into the portal vein of the right kidney used for micropuncture. The remaining blood vessels were ligated. The injection of lissamine green dye allowed to test if aortic and portal perfusion were adequate. Throughout the experiment the kidney

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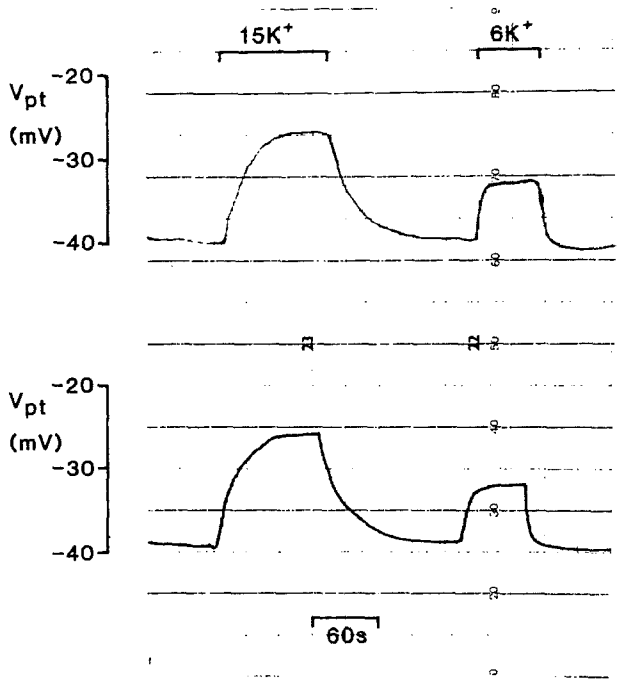

Fig. 1. Peritubular cell membrane potentials (V_{pt}) are measured simultaneously with two conventional microelectrodes in an individual proximal tubule. The distance between the two microelectrodes is about 100 μm. Please note that the magnitude of ΔV_{pt} to high K^+ is virtually identical in both cells. This is one of three similar experiments

was superfused. The perfusion solution had the following composition (in mmol/liter): 97 Na^+, 3 K^+, 1 Ca^{2+}, 1 Mg^{2+}, 92 Cl^-, 10 HCO_3^-, 1 $H_2PO_4^-/HPO_4^{2-}$, 5 glucose and 3 glycine. pH was maintained at 7.8 by equilibrating the solution with 1% CO_2 (99% air). Different K^+ concentrations of the solutions were obtained by using an equimolar substitution of KCl for NaCl. DIDS (4,4'-diisothiocyanostilbene-2,2'disulfonic acid) was added at a concentration of 5×10^{-4} mol/liter to the peritubular perfusate if applicable. The peritubular perfusion could be changed within seconds from control to experimental perfusates and vice versa.

Measurements of Cell Membrane Potential and Intracellular pH

Peritubular cell membrane potentials (V_{pt}) were measured with conventional microelectrodes filled with 1 mole/liter KCl in reference to a grounded Ag/AgCl macroelectrode. The conventional microelectrodes were connected via Ag/AgCl half-cells to a high impedance electrometer (FD 223, W.P. Instruments, Hamden, Conn.). The voltage signals were recorded by a two-channel pen-recorder (Linseis, Selb, FRG).

The pH-sensitive, liquid-ion exchanger microelectrodes were manufactured as described in previous reports [2, 26]. The calibration solutions contained 20 mmol/liter HEPES and 90 mmol/liter KCl, titrated with 0.1 mole/liter NaOH either to pH 7.8 or to pH 6.8. Electrode tips were about 1 μm, the slopes of the pH-sensitive electrodes were between 50 and 59 mV for a 10-fold change of the H^+ concentration. The resistances of the pH-sensitive microelectrodes were between 2×10^{11} and 4×10^{11} Ω.

The conventional and pH-sensitive microelectrodes were inserted into two different cells (100 μm apart from each other) of the same tubule. This experimental approach seemed adequate since in proximal tubule of frog kidney, cells are electrically well coupled. Thus cell membrane potentials are identical in different cells of the same tubule portion. Figure 1 is a typical recording showing virtually the same membrane potentials and the identical responses to high K^+ traced by two conventional microelectrodes in different cells of the same tubule (distance between microelectrodes approx. 100 μm). pH_i was calculated from equation:

$$pH_i = pH_o - (V_{pt}^H - V_{pt})/S.$$

The pH_o is the extracellular pH ($pH_o = 7.8$), V_{pt}^H is the peritubular H^+ electrochemical potential difference, the symbol S is the electrode slope.

II. Experiments in Fused Proximal Cells

Fusion of renal epithelial cells *within* an isolated tubule portion was described in the preceeding paper and in a recent report [11, 18]. The fused tubules were transferred on a glass coverslip and superfused via a multichannel pipette system [11]. HCO_3^--containing and HCO_3^--free solutions with low Cl^- concentrations were applied. Both solutions were composed similar to the perfusion solution for intact tubules (vide supra) except that NaCl was substituted by Na-gluconate and that HEPES (10 mmol/liter) was used in the HCO_3^-/CO_2-free perfusate. Since Ca^{2+} ions bind to gluconate$^-$, $CaCl_2$ was added under the control of a Ca^{2+}-sensitive macroelectrode to obtain the same Ca^{2+} activity present in the gluconate$^-$-free perfusion solution.

Voltage-Clamp Measurements

Fused tubules were impaled with three microelectrodes. One conventional microelectrode (1 mole/liter KCl) was used to inject negative current (1 to 20 $\times$ 10^{-9} A) and to clamp the cell membrane potential to various levels, another one was used to monitor the cell membrane potential (V_m) and a third one, a pH-sensitive microelectrode, was applied to measure pH_i when the potential was increased stepwise to more (cell inside) negative potentials. High-impedance amplifiers and a current-injecting device (constant current delivery: 0.5 to 100 $\times$ 10^{-9} A; Frankenberger electrometer, Germering, FRG) were used to trace V_m and pH_i.

Statistics

Data are indicated as mean values ± standard error (SEM). Number of observations is given in parentheses. Paired students t-test was used to compare the data and to estimate the significance of differences. Significantly different is $P < 0.05$ or less.

Results

Experiments in Intact Tubules

Figure 2 represents two original recordings that show the effect of high peritubular K^+ concentra-

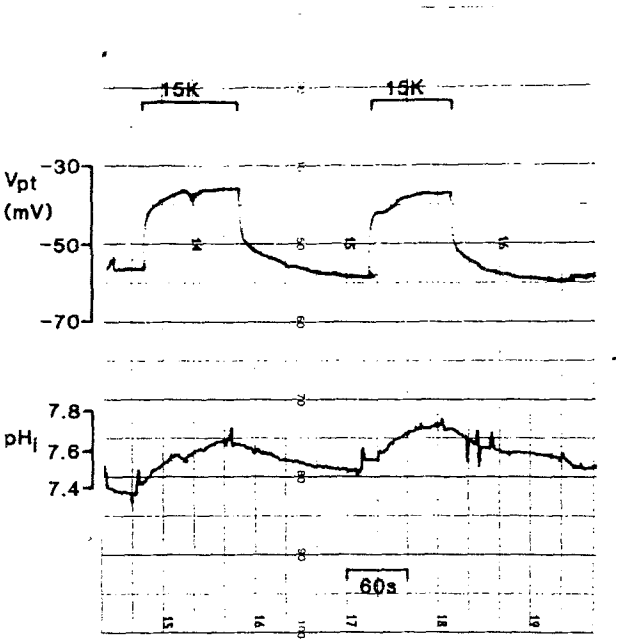

Fig. 2. Effect of hyperkalemia on V_{pt} and pH_i. The bar "15 K^+" indicates that the peritubular perfusate contains 15 mmol/liter K^+ in contrast to the control solution (3 mmol/liter)

Table 1. Cell membrane potential and intracellular pH in frog proximal tubule[a]

	Control	Δ	15 K^+	n
V_{pt}	−55.3 ± 2.4	17.3 ± 1.8	−38.0 ± 1.2	11
pH_i	7.54 ± 0.02	0.12 ± 0.01	7.66 ± 0.02	11

[a] Peritubular cell membrane potential (V_{pt}, mV) and intracellular pH (pH_i) in control conditions (3 mmol/liter K^+) and after application of 15 mmol/liter K^+. Results are mean values ± SEM. All Δ-values of these paired experiments are significantly different from zero.

tion on V_{pt} and pH_i. Two individual cells of the same tubule were impaled with a conventional and a pH-sensitive microelectrode. The increase of peritubular K^+ concentration causes a depolarization of the cell membrane potential and concomitantly an increase of pH_i. This effect is fully reversible. Figure 3 shows the individual experimental values of this series. Increasing the K^+ concentration of the peritubular perfusate from 3 to 15 mmol/liter depolarizes V_{pt} from −55 ± 2 to −38 ± 1 mV. The mean values are summarized in Table 1. Intracellular pH is 7.54 ± 0.02 in the control period and increases to 7.66 ± 0.02 in the experimental (high K^+) period. Figure 4 displays the relationship between the change of cell voltage and the corresponding change of pH_i. The Figure includes data obtained with various peritubular K^+ concentrations (1, 6, 9

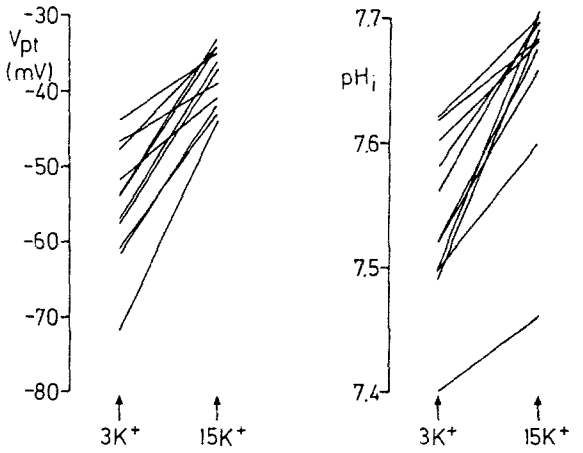

Fig. 3. Response of V_{pt} and pH_i on hyperkalemia in individual cells of the proximal tubule

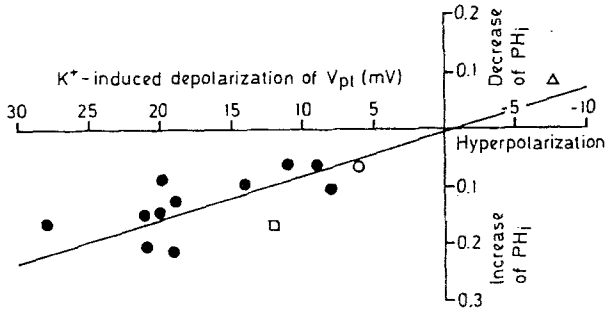

Fig. 4. Relationship between the change of the cell membrane potential (V_{pt}) and intracellular pH (pH_i). $y = 0.0087 + 0.0063x$; corr. coeff. = 0.94. ● = 15 mmol/liter K^+; □ = 9 mmol/liter K^+; ○ = 6 mmol/liter K^+; Δ = 1 mmol/liter K^+

and 15 mmol/liter K^+). Note the linear relationship between pH_i and cell membrane potential. Decreasing V_{pt} by 15 mV acidifies pH_i by about 0.1 pH units ($y = 0.012 + 0.0068x$; corr. coeff. = 0.81). DIDS is a stilbene derivative known to block anion transport in different tissues. Experiments in four tubules show that, in the *absence* of DIDS, an increase of peritubular K^+ concentration from 3 to 15 mmol/liter depolarizes the cell membrane potential by 20 ± 1 mV (from 57 ± 2 to −37 ± 1 mV) and increases intracellular pH from 7.56 ± 0.02 to 7.70 ± 0.02 (Table 2). The application of 5×10^{-4} mole/liter DIDS for 30 min hyperpolarizes the cell membrane potential from −57 ± 2 to −71 ± 4 mV (Table 2). As shown in Fig. 5 and Table 2, DIDS almost eliminates the high K^+-induced increase of pH_i. In contrast to the lack of change in pH_i, K^+-induced depolarization of cell membrane is significantly increased after application of the stilbene derivative.

Table 2. Cell membrane potentials and intracellular pH in frog proximal tubule before and after application of DIDS[a]

		3 K^+	Δ	15 K^+
Control	V_{pt}	−56.5 ± 2.2	20.0 ± 0.4[b]	−36.5 ± 1.3
	pH_i	7.56 ± 0.02	0.14 ± 0.03[b]	7.7 ± 0.02
DIDS	V_{pt}	−70.8 ± 3.9	25.5 ± 2.9[b]	−45.3 ± 1.0
	pH_i	7.70 ± 0.03	0.01 ± 0.01	7.71 ± 0.02

[a] Peritubular cell membrane potential (V_{pt}, mV) and intracellular pH (pH_i) at 3 and 15 mmol/liter K^+ in control conditions and after application of 5×10^{-4} mmol/liter DIDS.
[b] Indicates a significant difference between the 3 K^+ and 15 K^+ values. Results are mean values ± SEM.

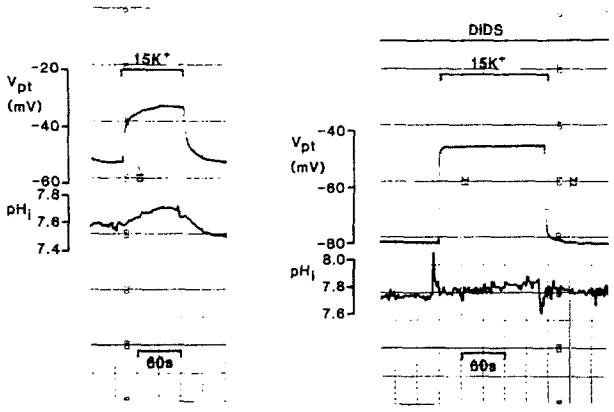

Fig. 5. Effect of high K^+ on V_{pt} and pH_i before and during DIDS treatment. The time difference of the two recordings was 30 min. The bar "15 K^+" indicates that the perfusion solution was switched from 3 to 15 mmol/liter K^+. Similar recordings were obtained in another four cells

Experiments in Fused Cells

Whereas cell membrane *depolarization* can be performed easily by increasing extracellular K^+ concentrations, sustained cell membrane *hyperpolarization* above the resting cell membrane potential can be maintained only by voltage-clamp techniques. Since fused tubules have diameters of at least 100 μm, intracellular impalements with several microelectrodes in the same cell are feasible. Figure 6 shows some original recordings obtained in fused cells in presence and absence of the CO_2/HCO_3^- buffer system. When V_m is hyperpolarized stepwise, starting from the individual resting potential, there is a concomitant change of pH_i. Please note, that with both open (CO_2/HCO_3^-) and closed (HEPES) buffer systems there is intracellular acidification induced by the increase of V_m. However, the pH_i response is more marked in presence of the CO_2/HCO_3^- buffer system. Quite often, we observed that pH_i measurements in the CO_2/HCO_3^- series were "electrically noisy" at high intracellular voltages (*see* Fig. 6). We assume that this is caused by extremely high specific cell membrane resistances which are likely to occur at acidic intracellular pH (decrease of K^+ conductance). Under these conditions the whole cell membrane conductance is composed of the remaining, rather small conductances for K^+ and HCO_3^- (the latter is low due to reduced intracellular HCO_3^- at acidic pH_i). Figure 7 shows an original tracing with increased time resolution. Induced by the hyperpolarizing current the cell cytoplasm acidifies within 5 to 10 sec. The time course reflects indeed the "real" pH_i change since the response time of the pH-sensitive microelectrode is in the range of 1 sec (95% of full response when changing pH for one unit). Figure 8 summarizes the results of the voltage-clamp experiments. There is a linear relationship between V_m and pH_i in both CO_2/HCO_3^--buffered ($y = 0.0074 + 0.0148x$; corr. coeff. = 0.98) and HEPES-buffered ($y = 0.0087 + 0.0063x$; corr. coeff. = 0.94) solutions. However, the intracellular H^+ activity increases by more than twice in a CO_2/HCO_3^--buffered cell compared to the experiments applying HEPES buffer in the extracellular fluid.

Discussion

We observed that in frog *proximal* tubule intracellular pH depends on cell membrane potential. In frog *early distal* tubule it was shown that the cell membrane depolarization, induced by high K^+ or by the addition of Ba^{2+} to the perfusate, leads to cytoplasmic alkalinization. A furosemide-induced hyperpolarization of the cell membrane potential causes a decrease of pH_i [25]. Ouabain which depolarizes the cell membrane potential induces intracellular alkalosis in frog proximal tubule [17, 26]. A transient depolarization due to the addition of organic substrates to the lumen fluid is paralleled also by a transient intracellular alkalinization in cells of frog proximal tubule [14]. The explanation of the high K^+-induced increase of intracellular pH is most likely based on the fact that peritubular HCO_3^- transport is rheogenic and thus voltage-sensitive [6, 11]. It is a Na^+/HCO_3^- cotransport system that transfers net negative charge from the cell to the blood (due to a coupling ratio of Na^+/HCO_3^- less than one). This view is supported by the finding that DIDS like SITS, known to inhibit HCO_3^- exit [4, 5, 15], abolishes the K^+-induced increase of pH_i although—after application of DIDS—the amplitude of depolarization induced by high K^+ is even larger as compared to the control experiment. This large response of the cell membrane potential to high K^+

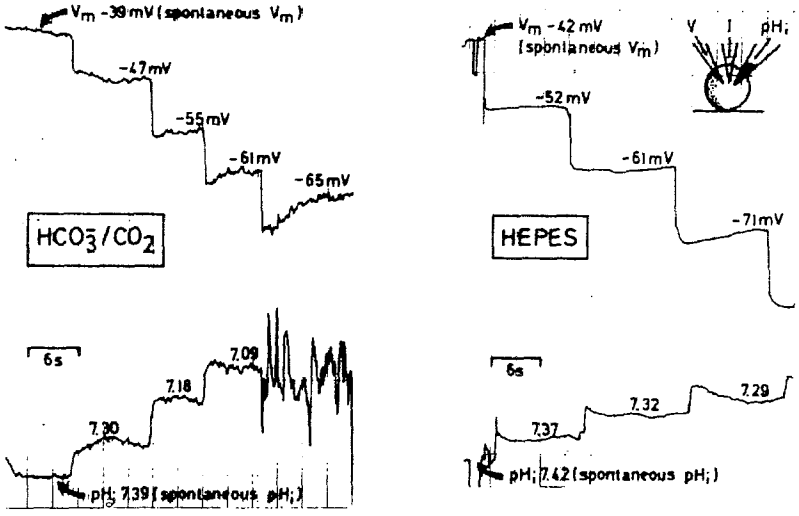

Fig. 6. Intracellular pH measurements in fused proximal tubules while the cell membrane was clamped to various voltages (*see inset* at upper right corner). The cell membrane as hyperpolarized stepwise starting from the resting cell membrane potential (spontaneous V_m). The extracellular medium contained either CO_2/HCO_3^- buffer or HEPES buffer. Please note that with increasing cell membrane potential intracellular acidification is much more pronounced in the presence of the CO_2/HCO_3^- buffer system as compared to the experiment with HCO_3^--free, HEPES-buffered solution

solutions in *presence* of DIDS is most likely caused by an increase of the relative K^+ conductance of the basolateral membrane after inhibition of rheogenic HCO_3^- transport. This assumption is consistent with the cell membrane hyperpolarization observed after addition of anion-transport blockers [4, 24]. Note that DIDS increases pH_i to a similar extent as high K^+ perfusion does in *absence* of DIDS. Thus, inhibition of the peritubular HCO_3^- exit step either by reducing the electrochemical driving force (e.g. by depolarization of the cell membrane potential) or blockade of the transport system *per se* leads to the same phenomenon, namely intracellular alkalinization. Recently, it was observed that in rat proximal tubule, cell membrane depolarization, induced by high K^+ or Ba^{2+}, leads also to intracellular alkalosis. This cytoplasmic alkalinization can be abolished by removal of sodium from the peritubular perfusion [1]. In this particular segment of the rat kidney a rheogenic Na^+/HCO_3^- cotransporter located in the basolateral cell membrane has been indeed identified [28]. Removal of Na^+ can inhibit the Na^+/HCO_3^- cotransporter [7] and thus renders pH_i *insensitive* to variations of cell voltage. This mechanism could at least in part explain the clinical observation that hyperkalemia is usually associated with systemic acidosis. More than 80% of bicarbonate filtered in the glomerulus is reabsorbed by the proximal convoluted tubule [23]. An increase in plasma K^+ concentration will depolarize the membranes of proximal tubule cells and thus increase pH_i. Since the Na^+/H^+ exchanger located in the luminal cell membrane is sensitive to intracellular pH [3], an intracellular alkalosis will inhibit the Na^+/H^+ exchanger and consequently decrease the rate of H^+ secreted into the tubule fluid. Such a mechanism exists in frog early distal tubule [25] and most likely

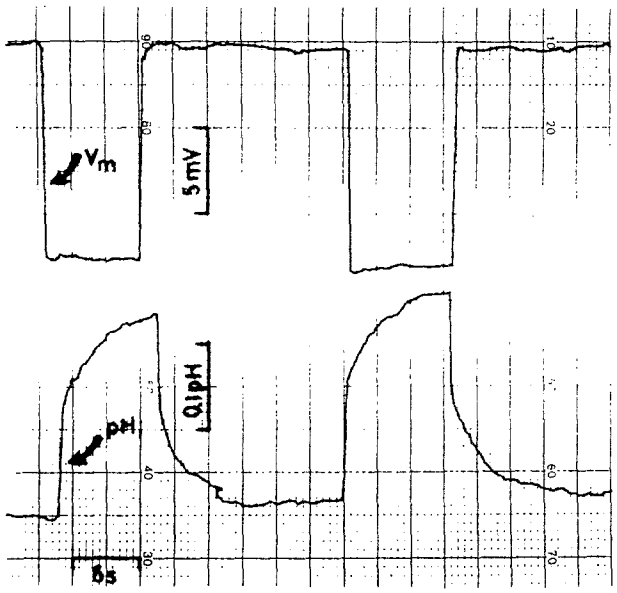

Fig. 7. Relationship between intracellular voltage (V_m) and pH_i in fused proximal tubule (120 μm). Hyperpolarization of V_m by 15 mV acidifies pH_i by 0.23 pH units. This experiment was done in presence of the CO_2/HCO_3^- buffer system. In this record the new steady-state pH_i is approached within 6 to 6 sec. This time course, however, is critically dependent on cell size (more specifically, on cell surface-to-cell volume ratio). In the intact epithelium (cells size 25 μm), pH_i changes are expected to be much faster

occurs in proximal tubule cells (Fig. 9). In rat kidney, peritubular application of Ba^{2+} reduces the rate of HCO_3^- reabsorption significantly [8].

Experiments with three microelectrodes inserted simultaneously into a renal cell is only feasible in "artificially enlarged" fused cells. Such cells exhibit both luminal and peritubular cell membrane properties [20] and can be held under voltage clamp

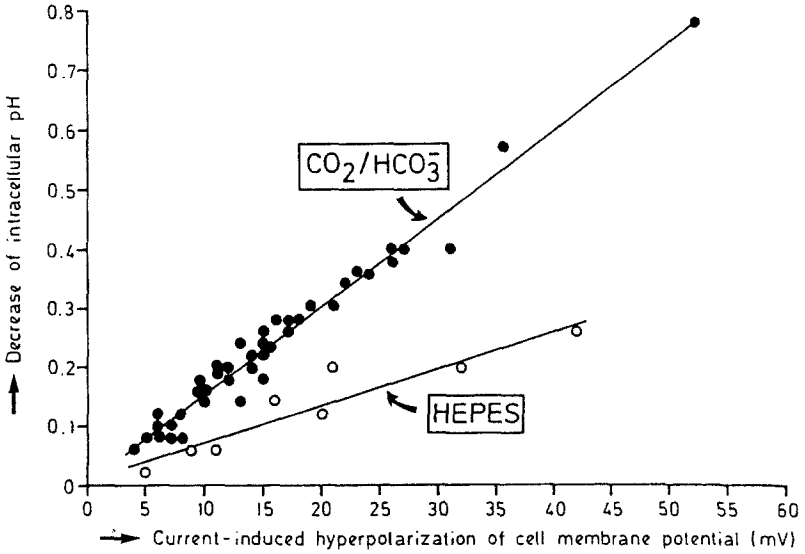

Fig. 8. Voltage-clamp experiments in fused proximal tubule. Current-induced hyperpolarization of cell membrane potential acidifies the cell cytoplasm. In CO_2/HCO_3^- buffer: $y = 0.0074 + 0.0148x$; corr. coeff. = 0.98. In HEPES-buffer: $y = 0.0087 + 0.0063x$; corr. coeff. 0.94

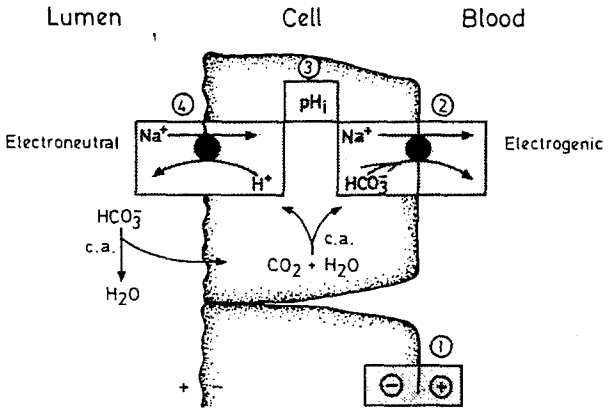

Fig. 9. Role of cell voltage in the regulation of HCO_3^- transport. An intracellular potential change (first event) alters the electrochemical driving force of the rheogenic, Na^+-dependent HCO_3^--transport system (coupling ratio of $Na^+/HCO_3^- < 1$) in the peritubular cell membrane (second event). HCO_3^- ions will be redistributed across the cell membrane and pH_i (third event) will approach a new steady state. The activity of the Na^+/H^+ exchanger depends critically on pH_i since the structural conformation of this transport protein is altered by H^+ ions (fourth step). Although this antiporter is electrically neutral, its activity will finally depend on intracellular voltage. Thus, H^+ ion secretion (or HCO_3^- reabsorption) in proximal tubule is voltage dependent due to the rheogenicity of the HCO_3^--transport system

while intracellular pH is measured. Using this experimental model we observed—similar to the intact tubule preparation—a clear linear relationship between cell voltage and pH_i. Hyperpolarizing current leads to cell acidification. This effect is pronounced when HCO_3^- ions are present in millimolar concentrations in the extracellular (and thus also in the intracellular) medium and is best explained by the fact that the proximal tubule cell possesses a specific HCO_3^- transport system which shuttles more HCO_3^- ions across the cell membrane if HCO_3^- is available at high (physiological) concentrations. Another observation deserves our attention. pH_i is altered significantly more by hyperpolarization than by depolarization, starting from the individual spontaneous cell membrane potentials. This could be caused by the application of different experimental models (intact tubule *vs.* fused cell) and/or by different voltage-clamp techniques (K^+-induced depolarization *vs.* current-induced hyperpolarization). However, it could also reflect a regulatory (voltage-dependent) component of intracellular pH homeostasis. The fact that the cell cytoplasm is significantly acidified in the virtual *absence* of the HCO_3^-/CO_2 buffer-system is also consistent with a finite OH^- or H^+ conductance of the cell-membrane as discussed recently for proximal tubule of rat kidney [9].

In snail neurons an increase of the H^+ ion permeability of the cell membrane was observed when cells were depolarized with voltage-clamp techniques [16, 22]. Such a mechanism would allow H^+ ions to enter the cell cytoplasm along the electrical field (i.e the cell membrane potential) and thus could buffer HCO_3^- ions accumulated in the depolarized cell. Whether such a mechanism is operative in renal cells is yet unknown.

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