Role of CFTR and CIC-5 in Modulating Vacuolar H⁺-ATPase Activity in Kidney Proximal Tubule

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Key Words

H⁺-ATPase • Chloride • CFTR • CIC-5 • Proximal tubule

Abstract

Background/Aims: 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 performed by H⁺-ATPase into subcellular vesicles. Largely known in intracellular compartments, this mechanism is also important at the plasma membrane of cells from various tissues, including kidney. The present work was performed to study the modulation of plasma membrane H⁺-ATPase by chloride channels, in particular, CFTR and CIC-5 in kidney proximal tubule. **Methods and Results:** Using *in vivo* stationary microperfusion, it was observed that ATPase-mediated HCO₃⁻ reabsorption was significantly reduced in the presence of the Cl⁻ channels inhibitor NPPB. This effect was confirmed *in vitro* by measuring the cell pH recovery rates after a NH₄Cl pulse in immortalized rat renal proximal tubule cells, IRPTC. In these cells, even after abolishing the membrane potential with valinomycin, ATPase activity was seen to be still dependent on Cl⁻. siRNA-mediated CFTR

channels and CIC-5 chloride-proton exchanger knockdown significantly reduced H⁺-ATPase activity and V-ATPase B2 subunit expression. **Conclusion:** These results indicate a role of chloride in modulating plasma membrane H⁺-ATPase activity in proximal tubule and suggest that both CFTR and CIC-5 modulate ATPase activity.

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Introduction

V-ATPases are ATP-dependent proton pumps, organized in multi-subunit complexes, which couple the energy released from ATP hydrolysis with the active extrusion of protons from the cytoplasm into the lumen of organelles or to the extracellular space. In endomembranes, V-ATPases are responsible for the acidification of the vesicular interior, which warrants an acidic intraorganellar pH [1-4]. In addition, H⁺-ATPases expressed in the plasma membrane transport protons to the outside of the cell, which is intricately related to

specialized cell function, as exemplified in osteoclasts [5, 6], renal tubular cells [7-9], inner ear [10, 11], epididymis and vas deferens [12, 13].

H⁺-ATPases can be regulated through a number of mechanisms, including counterion conductance [3, 4]. Although not directly affecting the proton ATPase activity, electrogenic translocation of protons across membranes by H⁺-ATPase creates a lumen-positive voltage in the absence of a neutralizing current, creating an electrochemical potential gradient that ultimately antagonizes inward transport of protons by H⁺-ATPase. This potential difference (PD) established by the gradient created by the H⁺-ATPase is normally dissipated by a parallel and passive Cl⁻ movement, which provides an electric shunt compensating for the positive charge transferred by the proton ATPase [3, 14]. The mechanism has been shown in most intracellular organelles that are acidified by proton ATPases, including lysosomes [15], endosomes [16-18] and trans-Golgi network [19, 20]. In addition, chloride dependence of plasma membrane H⁺-ATPase has also been observed in a number of tissues, including kidney and bone [8, 21-23]. However, due to the presence of numerous other conductances or electrogenic transporters in both apical and basolateral membrane domains, regulation of membrane H⁺-ATPase by chloride ions is difficult to study.

In the kidney, the role of Cl⁻ channels or conductances in modulating H⁺-ATPase activity has been observed in endosomal fractions [24, 25], brush border membrane vesicles [26, 27] and also in apical membranes of distal tubules [22, 28]. In isolated rabbit S3 proximal tubules (PT), the apical insertion of vesicles containing vacuolar H⁺-ATPases was found to be dependent on chloride, showing a significant delay in the absence of the anion [8]. In addition, in superficial S1/S2 segments of rat proximal tubules, H⁺-ATPase activity was also observed to be reduced after preincubation in Cl⁻ free solution [29].

The molecular identity of the associated Cl⁻-conducting proteins involved in the regulation of plasma membrane H⁺-ATPase has not been defined. Some candidates have been suggested, including CIC family members and the cystic fibrosis transmembrane conductance regulator (CFTR).

In proximal tubule, a number of studies have demonstrated an intracellular expression of CIC-5 colocalizing with the vacuolar H⁺-ATPase in the apical compartment [30]. Furthermore, mutations in the gene *CLCN5*, which have been shown to decrease or abolish CIC-5 activity, impair endosomal acidification and result

in low molecular weight proteinuria, hypercalciuria and kidney stones, characterizing Dent's disease [31]. It is possible, that CIC-5 is not the only chloride-conducting protein (conductance) playing a significant role in H⁺-ATPase activity in endosomes and brush border membrane from proximal tubules. CIC-5 lacks some features, for example activation by PKA (since chloride conductance in endosomes from proximal tubules is stimulated by this kinase), suggesting that another conductance could also be involved [3].

The positive modulation of CFTR (cystic fibrosis transmembrane regulator) channels by cAMP is well known [32]. This channel is expressed in renal endosomes, membranes of proximal and distal tubules, and intercalated cells of collecting ducts, especially in type- β cells [33]. Barasch and colleagues have demonstrated a defective acidification of intracellular organelles in cystic fibrosis, indicating a possible role for CFTR in organellar acidification dependent on V-type H⁺-ATPase [34]. In contrast, other authors failed to observe changes in the acidification rates of several intracellular organelles after activation of chloride currents stimulated by cAMP [35]. Thus, the role of CFTR in the activity of H⁺-ATPase remains still controversial.

The purpose of this work was to study the modulation of plasma membrane H⁺-ATPase by chloride channels in proximal tubule. In particular, V-ATPase activity was accessed in PT cells in which siRNA-mediated CFTR channel and chloride-proton exchanger CIC-5 knockdown was employed.

Materials and Methods

In vivo stationary microperfusion

The microperfusion procedure was previously described in detail [36]. Briefly, male Wistar rats (180 to 250g) were anesthetized with tiletamine + zolazepan (both at 30 mg/kg) and the left jugular vein was cannulated for infusion of a modified saline solution (140 mM NaCl, and 3% mannitol), at a rate of 0.1 ml/min. The left kidney was immobilized in a plastic cup, exposed by a lumbar approach and prepared for *in vivo* micropuncture. Proximal tubules were punctured by means of a double-barrelled micropipette, one barrel being used to inject FDC-green colored Ringer perfusion solution, and the other to inject Sudan-black colored castor oil, the latter used to block the injected fluid columns in the lumen. To measure luminal pH, proximal tubules (identified by the colored perfusion and its transepithelial PD) were impaled by a double barrelled asymmetric microelectrode, the larger barrel containing at its tip the H⁺-ion sensitive ion-exchange resin after silanizing with hexamethyldisilazane (both from Sigma Fluka, Buchs,

Switzerland) and the smaller barrel containing the reference solution (1M KCl) coloured by FDC-green. The microelectrodes were standardized after every perfusion by covering the isolated kidney “*in vivo*” with 100 mM NaCl/20 mM Na phosphate at pH 6.5, 7.0 and 7.8 into which the electrodes were immersed. By continuously measuring the luminal pH towards the steady-state level, the rate of tubular acidification, representing bicarbonate reabsorption, was evaluated in a solution, isolated and blocked by oil.

Thereby, luminal HCO_3^- activity, starting at 25 mM (perfusion solution) was progressively reduced to a stationary level by H^+ secretion. The voltage between the microelectrode barrels, representing luminal H^+ activity, was continuously recorded by means of a microcomputer equipped with an AD converter (Lynx, São Paulo, Brazil). Luminal bicarbonate was calculated from luminal pH and arterial blood PCO_2 and the rate of tubular acidification expressed as the half-time of the exponential reduction of the injected HCO_3^- concentration to its stationary level ($t_{1/2}$). Then, net HCO_3^- reabsorption (JHCO_3^-) per cm^2 of tubule epithelium was calculated by using the following equation:

$$\text{JHCO}_3^- = \frac{\ln 2}{t_{1/2}} \left[(\text{HCO}_3^-)_0 - (\text{HCO}_3^-)_s \right] \times \frac{r}{2}$$

Where $t_{1/2}$ is the half-time of bicarbonate absorption, r is the tubule radius and $(\text{HCO}_3^-)_0$ and $(\text{HCO}_3^-)_s$ are the concentrations of the injected HCO_3^- and HCO_3^- at the stationary level, respectively.

Cell model

IRPTCs were kindly provided by Dr. J. R. Ingelfinger (Pediatric Renal Research Laboratory, Massachusetts General Hospital, Boston, MA) and used from passages 33 to 43. Serial cultures were maintained in DMEM supplemented with 45 mM NaHCO_3 , 25 mM HEPES buffer, 0.1 mM sodium pyruvate, 0.01 mM nonessential amino acids, 5% (v/v) heat-inactivated fetal bovine serum, 100 IU/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin. All these solutions were purchased from GIBCO (Grand Island, NY). Cells were grown at 37°C , 95% humidified air-5% CO_2 (pH 7.4) in a CO_2 incubator (Lab-Line Instruments, Melrose Park, IL, USA). The cells were harvested with trypsin-ethylene glycol tetraacetic acid (0.02%), seeded on sterile glass coverslips or 6/24-well plates and incubated again for 72 h in the same medium to become partially confluent.

Vector-based small interfering RNA to CFTR channels and chloride-proton exchanger CIC-5

Short hairpin (sh) constructs to CFTR and CIC-5 were designed, generated, and introduced in the pRNA-U6/Neo and pRNAT-CMV3.2/Neo vectors, respectively, by GenScript (Piscataway, NJ). The sequences for siRNA for CFTR were TGT GTC TGT AAA TTG ATG GCC and ATA CCT TCG ATA TTT CAC GCT and for CIC-5, ATA ATC AGT GAG ACC ACG TAC AGC GGC TTT CCG GTG GTG GTG TCC CGA GAG TCA CAA AGA. The plasmids were subsequently transformed into XL1-blue competent cells (Stratagene, La Jolla, CA) by the calcium chloride method [37] and were purified for transfections

by using Wizard® Plus Maxipreps DNA Purification System from Promega (Madison, WI).

Transfections with siRNAs constructs were performed with Lipofectamine 2000 (Invitrogen, Rockville, MD) in 6-well plates following manufacturer's protocol. For stable transfection, after 24 hours of transfection, cells were transferred to the selective medium containing Geneticin (500 $\mu\text{g}/\text{mL}$, Invitrogen). Single cell clones were selected and propagated in selective medium after 2 weeks of incubation.

Measurement of intracellular pH by fluorescence microscopy

The dual-excitation (440 vs 490 nm), single emission (530 nm) ratiometric technique with the pH-sensitive fluorophore BCECF (Molecular Probes, Eugene, OR) was used to measure intracellular pH at 37°C , as previously detailed [38]. Briefly, cells grown to confluence on glass coverslips were loaded with the dye by exposure for 5 min to 10 μM BCECF-AM in the control solution and placed in a thermoregulated chamber mounted on an inverted epifluorescence microscope (Nikon, TMD). Extracellular dye was washed away with the control solution and subsequently, cells were prepulsed with 20 mM NH_4Cl during 2 minutes for acid loading. Then, the glass coverslips were rinsed with Na^+ -free solution with or without inhibitors, as described later, to measure H^+ -ATPase activity. The fluorescence was monitored alternately using 440 nm (pH-insensitive) or 490 nm (pH-sensitive) as excitation wavelengths utilizing a xenon light source. Emission was measured at 530 nm by a photomultiplier-based fluorescence system (Georgia Instruments, PMT-4000) at time intervals of 5 s. $\text{pH}(i)$ was calculated from the fluorescence emission ratio of the two excitation wavelengths using a standard calibration procedure based on the use of 10 μM nigericin in high-potassium Ringer, varying pH values (between 5.9 and 8) [36]. In all experiments, performed in 0 Na^+ medium, the pH recovery was initially near zero, and then started to increase at a rate considerably slower than that observed in Na^+ Ringer's solution. This Na^+ -independent recovery rate was measured for 2 min after its start, then calculated by linear regression analysis and considered to be the rate due to H^+ extrusion by H^+ -ATPase (dpH/dt , pH units/min), as described previously [8]. Na^+ -control solution contained 141 mM NaCl, 5.4 mM KCl, 1 mM CaCl_2 , 0.4 mM KH_2PO_4 , 0.5 mM MgCl_2 , 0.4 mM MgSO_4 , 0.5 mM Na_2HPO_4 , 0.6 mM glucose and 10 mM HEPES (pH 7.4). Na^+ -free solution had similar composition except that NaCl was replaced by NMDG. For the Na^+/Cl^- -free solution, Cl^- was substituted by gluconate (5 mM K^+ gluconate, 7 mM Ca^{+2} gluconate (this high concentration was used due to the quenching of Ca^{+2} by gluconate) and 1 mM Mg^{+2} gluconate).

The osmolality of solutions ranged from 295 to 305 mosmol/kg H_2O as measured by an osmometer (Osmette, Precision Systems, Philadelphia, PA). The Na^+ concentration in nominally Na^+ -free solutions was less than 1 mEq/l. All other solutions employed in this work were prepared as described before [36] by using reagents from Sigma (St. Louis, MO) unless otherwise specified.

Intrinsic intracellular buffering power

The pH(i) dependence of intrinsic intracellular buffering capacity (β_i) was determined by means of the technique described by Boyarsky and colleagues [39]. β_i corresponds to the buffering power of all intracellular buffers excluding $\text{CO}_2/\text{HCO}_3^-$. Therefore, estimations of β_i were carried out by using Hepes-buffered solutions. In addition, H^+ transport mechanisms were kept inhibited throughout the procedure by 0 mM Na^+ solution plus 0.1 μM concanamycin. After removal of Na^+ from the external medium, the cells were exposed to a HEPES-buffered solution containing 50 mM NH_4Cl , which was then stepwise reduced to 1 mM (in 50, 20, 10, 5, 2.5, and 1 mM steps). Calculation of β_i was performed according to the formula $\beta_i = \Delta[\text{NH}_4^+]/\Delta\text{pH}_i$, where the intracellular NH_4^+ concentration ($[\text{NH}_4^+]_i$) was calculated from the Henderson-Hasselbalch equation on the assumption that $\text{NH}_{3i} = \text{NH}_{3o}$.

Cell surface biotinylation

In order to measure plasma membrane H-ATPase B2 subunit expression we used a surface biotinylation assay [9]. For this purpose, IRPTC cells grown to confluence in six-well plates were rinsed and then prepulsed with 20 mM NH_4Cl during 2 minutes. Subsequently this solution was substituted by a Na^+ -free solution alone or together with 10 μM NPPB, during 5 minutes. All of the following manipulations were performed at 4°C. The cells were then rinsed with ice-cold PBS-Ca-Mg (PBS with 0.1 mM CaCl_2 , 1.0 mM MgCl_2) three times. The surface membrane proteins were biotinylated by incubating the cells twice for 25 min with 2 ml of biotinylation buffer (150 mM NaCl, 10 mM triethanolamine, 2 mM CaCl_2 , and 2 mg/ml EZ-Link sulfo-NHS-SS-biotin). The cells were washed twice for 20 min with a quenching buffer (PBS-Ca-Mg/100 mM glycine) and then solubilized for 1 h by adding RIPA buffer (50 mM Tris; 150 mM NaCl; 1 mM EDTA; 0.5% Sodium Deoxycholate; 1% Triton X-100, pH 7.4) containing protease inhibitors (Protease inhibitor, Roche and 0.7 $\mu\text{g}/\text{ml}$ pepstatin A). The samples were centrifuged at 15,000 g for 10 min, and 50 μl of streptavidin-coupled agarose were added to the supernatants (1.5 mg of crude cell lysate). After 1 h of incubation, the beads were washed three times in solubilization buffer and then prepared for SDS-PAGE and immunoblotting.

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting were performed as previously described [40]. Briefly, proteins were first solubilized in sample buffer (10% SDS; 20% glycerol; 100 mM dithiothreitol; 2.9 mM Tris pH 6.8; 0.1% bromophenol blue) and then were immediately separated by SDS-PAGE by using 7.5% polyacrylamide gels according to Laemmli [41]. Proteins were then transferred from the gel to PVDF membranes (Immobilon-P; Millipore, Bedford, MA), that were sequentially incubated with blocking solution (5% nonfat dry milk and 0.1% Tween 20 in PBS, pH 7.4) for 1 h at room temperature. Then, the membranes were subjected to overnight incubation at 4°C with the primary antibodies, rabbit polyclonal anti-CFTR (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal anti-CIC-5 (1:500, Alpha Diagnostic International, San Antonio, TX), mouse monoclonal anti-H⁺-ATPase B2 subunit (1:500, Santa

Fig. 1. Role of chloride conductance in modulating H^+ -ATPase-mediated-bicarbonate reabsorption in proximal tubules *in vivo*. A. Kidney proximal tubules were perfused with control solution or with the NHE3 inhibitor S3226 (2 μM) alone or together with the chloride channel inhibitor NPPB (10 μM). The reduction in $J_{\text{HCO}_3^-}$ after inhibition of NHE3 was even more pronounced when Cl^- conductance was blocked, indicating a role of chloride ions in modulating the NHE3-independent- H^+ -ATPase-dependent component of net bicarbonate reabsorption. Number of perfused tubules is indicated in the bars. # $P < 0.05$ vs S3226, $^{\$}P < 0.005$ and $^{*}P < 0.001$ vs control. B and C. Representative experimental tracings of pH changes of the lumen of proximal tubules in the presence of S3226 (2 μM) alone or plus NPPB (10 μM), respectively. 1. Injection of 25 mM HCO_3^- -containing solution; 2. Rapid CO_2 -dependent-acidification; 3. Transport-dependent acidification (analyzed curve); 4. Stationary state.

Cruz Biotechnology, Inc., Santa Cruz, CA) or rabbit polyclonal anti-megalin (1:10000, gently provided by Dr. Daniel Biemesderfer, Yale University, New Haven, CT) in the same solution.

Luminal condition	[HCO ₃ ⁻]	t _{1/2}	pHs	J _{HCO₃}	n
Control	4.12±0.42	4.45±0.27	6.64±0.05	2.11±0.17	20
S3226	2.77±0.63	10.14±1.22*	6.41±0.10	1.14±0.19 ^s	8
S3226 + NPPB	5.28±0.82 [#]	14.24±1.1*	6.76±0.06	0.72±0.08 ^{#*}	10

Table 1. Means ± SE of stationary bicarbonate concentration ([HCO₃⁻]), half-time of acidification (T_{1/2}), stationary pH (pHs) and net bicarbonate reabsorption (J_{HCO₃}), during luminal perfusion of proximal tubules with control solution or S3226 (2 μM) together or not with NPPB (10 μM). Values for [HCO₃⁻] and t_{1/2} are in mM and s, respectively. [#]P < 0.05 vs S3226, ^sP < 0.005 and *P < 0.001 vs control.

After extensive washes, the membranes were incubated for 1h with the appropriated horseradish peroxidase-conjugated IgG secondary antibodies (Zymed Laboratories, San Francisco, CA). Membranes were again washed with blotto and finally rinsed with PBS. Bound antibody was detected with ECL-enhanced chemiluminescence (GE Healthcare, Piscataway, NJ) according to manufacturer's protocols. The visualized bands were digitized using the ImageScanner (GE HealthCare) and quantified by the ImageQuant program (Molecular Dynamics).

Immunofluorescence

IRPTC stably expressing siRNA against CFTR channels or chloride-proton exchanger CIC-5 were grown to partial confluence onto glass coverslips. After an ammonium pulse by means of 20 mM NH₄Cl and a recovery period of about 5 minutes, the cells were fixed for 10 min with paraformaldehyde 4% at room temperature. After several washes with PBS/Tween 0.1%, cells were blocked in PBS containing 2% BSA for 60 min at room temperature. The samples were then incubated with the primary antibody, anti-CFTR or anti-CIC-5 subunit (1:50, for both antibodies) for 18 h and washed three times with PBS/Tween 0.1%. Subsequently, the cells were incubated with Alexa Fluor 488 donkey anti-rabbit (Invitrogen) for 90 min. Again the cells were washed, stained with DAPI (4',6-diamidino-2-phenylindole), mounted onto glass slides and analyzed at room temperature by using laser excitation at 458 or 488 nm on a Zeiss LSM 510 real-time confocal microscope.

Results

In vivo modulation of H⁺-ATPase activity by chloride channels (conductance) in proximal tubule

H⁺-ATPase activity in renal proximal tubule was measured as the rate of bicarbonate reabsorption by means of stationary microperfusion *in vivo*. Since Na⁺/H⁺ exchanger isoform 3 (NHE3) represents the major H⁺ secretory mechanism involved in NaHCO₃⁻ reabsorption in proximal tubules [42, 43], H⁺-ATPase-dependent bicarbonate reabsorption was evaluated in the presence of 2 μM S3226, a specific NHE3 inhibitor. Figure 1A

shows that S3226 significantly inhibited the rate of bicarbonate flux (J_{HCO₃}) in luminally perfused proximal tubules (to 1.14±0.19 nmol/cm².s, n=8, in comparison to control, 2.11±0.17 nmol/cm².s, n=20, p< 0.005). Furthermore, this Na⁺-independent- mediated HCO₃⁻ reabsorption was seen to be even further reduced when the unspecific chloride channel blocker NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid) was used together with the NHE3 inhibitor (reaching 0.72±0.08 nmol/cm².s, n=10, p<0.05 vs S3226). Representative curves of pH changes of the lumen of proximal tubule in terms of mV in the presence of S3226 alone or together with NPPB (10 μM) are given in Fig. 1B and 1C.

As seen in Table 1, these changes in bicarbonate transport were followed by significant modifications in half-time of acidification (t_{1/2}) in all studied groups. Nevertheless, due to partial inhibition of H⁺-ATPase by NPPB, stationary bicarbonate concentration was significantly greater than that found for the S3226 group, indicating that the compensatory activity of H⁺-ATPase on HCO₃⁻ reabsorption is impaired after inhibition of chloride channels.

Taken together, these results suggest that chloride conductances modulate *in vivo* H⁺-ATPase activity in kidney proximal tubules.

Involvement of chloride conductance in the modulation of vacuolar H⁺-ATPase activity in vitro

In order to determine the role of chloride conductance in modulating *in vitro* V-ATPase activity, Na⁺-independent pH(i) recovery from an acid load in immortalized rat proximal tubule cells (IRPTC) was evaluated by means of the NH₄Cl pulse technique. As observed previously [36], IRPTCs express both, Na⁺/H⁺ exchanger and vacuolar H⁺-ATPase as H⁺ extrusion systems. To obviate the contribution of Na⁺/H⁺ exchanger, in all experiments, intracellular pH recovery from the acid

Fig. 2. Influence of Chloride conductance in modulating Na^+ -independent- H^+ -ATPase-mediated activity in rat kidney proximal tubule cells. Typical tracings for control, Na^+ -free-treated cells without (A) or plus 10 NPPB μ M (B). After cellular acidification by means of the NH_4Cl pulse technique in IRPTCs, the initial fall in pH_i was followed by a slow recovery of pH_i in Na^+ -free solution-treated cells. The pH recovery rates were even further reduced when experiments with $0Na^+/0Cl^-$ solution or Na^+ -free solution plus 10 μ M NPPB were performed, showing a dependence of H^+ -ATPase activity on chloride conductance. (C) The initial rates of pH recovery (pH units/min, first two minutes of pH recovery) were calculated from curves of all groups by linear regression analysis and expressed as J_{H^+} , through the relation: $J_{H^+} = \beta_1 \times dpH/dt$ (C). Number of experiments is indicated in the bars. * $P < 0.001$ vs 0 mM Na^+ .

Fig. 3. Importance of cell potential difference (PD) in H^+ -ATPase activity in IRPTCs. The effect of valinomycin (10 μ M), a potassium selective ionophore that reduces transmembrane potential in potassium-rich media (100 mM K^+) was evaluated by means of Na^+ -independent intracellular pH recovery by the ammonium pulse technique and expressed in terms of J_{H^+} . Data indicate that even after abolishing PD, H^+ -ATPase activity is still dependent on chloride channels. Number of experiments is indicated in the bars. * $P < 0.001$ vs 0 mM Na^+ and § $P < 0.005$ vs 0 mM Na^+ high K^+ /valinomycin + NPPB (10 μ M).

load was examined in the presence of Na^+ -free solutions. In this condition, recovery from the acid load starts after approximately 2 min, since the pH recovery depends on the intracellular targeting and translocation of H^+ -ATPase from vesicles to plasma membrane. For this reason, in all experiments, dpH_i/dt was measured starting 2 min after the introduction of Na^+ -free solution. Care was taken to maintain the same level of acidification after ammonium pulse in all studied groups, around 6.2 to 6.4. Therefore, in the absence of Na^+ , pH recovery rates reached 0.12 ± 0.017 pH units/min ($n=11$). The velocity of pH recovery was still more reduced when 10 μ M NPPB was added to the Na^+ -free solution, reaching 0.035 ± 0.0033 pH units/min, $n=12$. Figures 2A and 2B illustrate two representative experiments in which IRPTCs were bathed with a 0 mM Na^+ -solution alone or together with NPPB, respectively, after the ammonium pulse. Furthermore, in the presence of Na^+ -free solution plus 0.1 μ M concanamycin (a specific inhibitor of H^+ -ATPase), the Na^+ -independent pH recovery was almost completely inhibited (reaching 0.015 ± 0.005 pH units/min, $n=6$) confirming the involvement of vacuolar H^+ -ATPase in this Na^+/H^+ -independent recovery from acid load.

Fig. 4. Involvement of CFTR channels in modulating H^+ -ATPase activity in proximal tubule cells. A. Expression of CFTR was analysed by immunoblotting and actin was blotted as a loading control. CFTR siRNA suppressed CFTR, whereas there was no effect on actin expression. The relative abundance of CFTR was quantitated by densitometric analysis and the combined data from 3 experiments are represented as columns in a bar graph, shown in B. C. Immunofluorescence microscopy showing CFTR staining in control and CFTR-siRNA-transfected IRPTC cells. D. The initial rate of Na^+ -independent intracellular pH recovery after cellular acidification with the NH_4Cl pulse technique was measured in IRPTCs stably transfected with siRNA-containing plasmids (PO17455S-1 and PO17455S-2) targeting rat CFTR or nonsilencing siRNA (CTRL), showing a significant inhibition of H^+ -ATPase activity after CFTR knockdown. Number of experiments is indicated in the bars. * $p < 0.001$ vs CTRL. E. Blots from CFTR-siRNA transfected cells were stripped and reprobed with anti- H^+ -ATPase B2 subunit, showing a marked reduction in the expression of ATPase.

Since the net efflux of acid (acid extrusion rate, J_{H^+}) is the product of total intracellular buffering power (β_i , mM/pH unit) and the rate of pH(i) recovery, it was possible to transform the data originally obtained in the form of dpH/dt (pH units/min) into H^+ fluxes (mM/min) out of the cells. β_i in turn, was obtained at the mean pH at which dpH/dt was measured. In fact, as seen in Figure 2C, J_{H^+} was significantly slower in the group where $0Na^+$ plus $10 \mu M$ NPPB was employed. This reduction was even more pronounced when experiments with a $0Na^+/0Cl^-$ solution were performed (and the dpH/dt reached 0.020 ± 0.0049 pHunits/min, $n=8$). These findings confirm the *in vivo* experiments and suggest that also in proximal tubule cells in culture (IRPTC), H^+ -ATPase is modulated by chloride channels.

Importance of membrane potential in modulating H^+ -ATPase-dependent activity

The reduction of J_{H^+} observed at low chloride levels or in the presence of the chloride channel inhibitor NPPB is consistent with a decreased shunt of the potential difference (PD) established by H^+ -ATPase via the Cl^- ions. In addition, the transmembrane PD of the epithelial cells is expected to hyperpolarize due to H^+ secretion. We therefore evaluated the effect of valinomycin ($10 \mu M$), a potassium selective ionophore that reduces transmembrane potential in a potassium-rich medium ($100 \text{ mM } K^+$, substituting NMDG of the $0Na^+$ solution). Under these conditions, it is expected that cell PD falls to near 0, while J_{H^+} was decreased from 3.07 ± 0.45 mM/min, $n=14$ to 0.71 ± 0.16 mM/min, $n=12$

Fig. 5. Involvement of CIC-5 chloride-proton exchangers in modulating H^+ -ATPase activity in proximal tubule cells. A. Representative Western blot analysis for CIC-5 and actin expression in cell lysates from IRPTCs transfected with nonsilencing control siRNA or CIC-5 chloride channels siRNA. The relative abundance of CIC-5 was quantitated by densitometry and the combined data from 3 experiments are represented as columns in a bar graph, indicated in B. C. Immunofluorescence microscopy showing CIC-5 staining in control and in CIC-5-siRNA-transfected IRPTC cells. D. Initial rate of pH_i recovery following acute intracellular acidification in the absence of Na^+ in IRPTCs stably transfected with nonsilencing and with siRNA-containing plasmids P69535-4 targeting rat CIC-5, showing a significant inhibition of H^+ -ATPase activity after CIC-5 knockdown. Number of experiments is indicated in the bars. (* $p < 0.001$). E. Blots from CIC-5-siRNA transfected cells were stripped and reprobed with anti- H^+ -ATPase B2 subunit, showing a significant reduction in the expression of ATPase.

($p < 0.001$, Fig. 3). This reduction is compatible with the decrease in H^+ current that has been observed upon decrease in membrane PD, in membranes such as those of *Neurospora* [44] or in artificial membranes [45]. In *Neurospora*, H^+ -ATPase is the main contributor to overall membrane potential found in these cells (interior negative). Under the same conditions, the addition of NPPB caused a further reduction in H^+ secretion (see Fig. 3). Our observations suggest that in addition to chloride, H^+ transport by H^+ -ATPase depends on membrane potential.

Influence of siRNA-mediated knockdown of CFTR on H^+ -ATPase activity in IRPTC

In order to evaluate the influence of CFTR in modulating H^+ -ATPase activity in IRPTCs, experiments in CFTR-siRNA transfected cells were performed. To reduce CFTR expression, IRPTCs were stably transfected with pRNA-U6/Neo plasmids containing specific siRNA sequences to the referred chloride channel. IRPTC for controls were transfected with empty vectors and efficiency of knockdown was

Fig. 6. Role of chloride conductance in modulating trafficking of H⁺-ATPase B2 subunit to apical surface of IRPTCs. A. Cell surface biotinylated proteins were subjected to SDS-PAGE and immunoblotting. Western blot analyses were performed using an antibody against B2 subunit of H⁺-ATPase. Aliquots from the supernatants were saved and probed with actin, used as a control. B. The amount of B2 subunit was quantitated by densitometry, and the combined data from 3 experiments are represented as columns in a bar graph, indicating that NPPB treatment after ammonium pulse does not alter H⁺-ATPase B2 subunit expression on apical surface of IRPTCs. C. Representative Western blot analysis for megalin and actin expression in cell lysates from IRPTCs transfected with nonsilencing controls siRNA or CFTR/CIC-5 siRNAs. The relative abundance of megalin was quantitated by densitometry and the combined data from 3 experiments are represented as columns in a bar graph, indicated in D.

evaluated by immunoblotting in cell lysates by using polyclonal antibodies to these proteins. Figure 4A illustrates a representative immunoblot with CFTR-specific antibody of IRPTC lysates, collected on the same day of functional analysis, confirming a knockdown of CFTR in cells transfected with both siRNA-containing plasmids (see also the densitometric analysis, shown in Fig. 4B). The knockdown was further substantiated by immunofluorescence studies, indicating a weaker and more diffuse staining pattern in CFTR-siRNA cells (see Fig. 4C).

The specificity of CFTR siRNAs for CFTR knockdown was confirmed in IRPTC transfected with empty vector (CTRL), which did not inhibit CFTR expression (not significant vs non-transfected cells, data not shown), whose J_{H^+} reached similar values to those obtained from the 0 mM Na⁺ group (3.21 ± 0.18 mM/min, $n=10$). Figure 4D illustrates H⁺-ATPase activity in terms of J_{H^+} fluxes in cells transfected with pRNA-U6/Neo alone or together with specific CFTR siRNAs (PO17455S-1 and PO17455S-2), showing a marked inhibition of H⁺-ATPase activity (reaching 1.70 ± 0.019 mM/min, $n=6$ and 1.18 ± 0.026 , $n=7$, respectively). This effect appeared to be related with the degree of CFTR knockdown, as observed in the Figures 4A and B. These specific knockdowns suggest that correct expression of CFTR is required to control plasma membrane H⁺-ATPase activity in proximal tubule cells.

In order to investigate whether the decrease in H⁺-ATPase activity in IRPTCs is due to decreased expression of the H⁺ extrusion mechanism, we analyzed the B2 subunit H⁺-ATPase expression in CFTR-siRNA transfected cells. Interestingly, the reduction of vacuolar H⁺-ATPase activity in these cells was seen to be consistent with a decrease of B2 subunit H⁺-ATPase expression, as observed in Fig. 4E. These results suggest a functional interaction between H⁺-ATPase and CFTR chloride channels in modulating H⁺-ATPase activity and, in addition, the modulation of the B2 subunit of vacuolar H⁺-ATPase expression.

Role of siRNA-mediated silencing of CIC-5 on vacuolar H⁺-ATPase activity in IRPTC

To further demonstrate the role of CIC-5 chloride-proton exchangers in modulating H⁺-ATPase activity, IRPTCs were stably transfected with pRNAT-CMV3.2/Neo plasmid containing or not CIC-5-targeting siRNA (CTRL and P69535-4, respectively). siRNA-based CIC-5 knockdown efficiency was also evaluated by immunoblotting, represented in the Fig. 5A and confirmed by densitometric analysis shown in Fig. 5B. The silencing was also confirmed by immunofluorescence staining, shown in Fig. 5C.

As observed in Fig. 5D, the knockdown strategy induced a significant decrease in H⁺-ATPase activity in cells transfected with CIC-5-siRNA construct (reaching

J_{H^+} of 3.21 ± 0.13 mM/min, $n=17$ in comparison to nonsilencing CIC-5 plasmid of 2.06 ± 0.096 mM/min, $n=11$, $p < 0.0001$). Again, transfection with non-targeting siRNA had no effect on pH responses in comparison to the 0 Na^+ group (see Fig. 2).

As in the case of CFTR, downregulation of CIC-5 ultimately led to a reduction of B2-V-ATPase expression, as illustrated in the representative immunoblotting and densitometry shown in Fig. 5E.

Taken together these results suggest that in addition to CFTR channels, CIC-5 chloride-proton exchangers are also important modulators of H^+ -ATPase function in IRPTCs.

Role of chloride conductance in modulating trafficking of H^+ -ATPase B2 subunit to apical surface of IRPTCs

To evaluate if NPPB ultimately affects trafficking of V-ATPase-containing-vesicles to the apical membrane of IRPTCs, which, in turn, could lead to a reduction in the H^+ -ATPase activity, we performed surface protein biotinylation assays in NPPB-treated cells. As observed in the representative immunoblotting shown in the Fig. 6A, as well as in its densitometric analysis illustrated in the Fig. 6B, the acute treatment of IRPTCs with 10 μ M NPPB failed to affect the surface amount of B2 subunit.

To further evaluate if CFTR and CIC-5 downregulation somehow induce a trafficking defect in IRPTCs with loss of megalin expression, we performed experiments in CFTR or CIC-5-siRNA transfected cells. The silencing of both CFTR and CIC-5 seem not to alter the total expression of megalin, as illustrated in Fig. 6C and D.

Discussion

It has been accepted that chloride ions moving along chloride conductances act to dissipate the electrical gradient established by the electrogenic transport of H^+ ions performed by H^+ -ATPase into subcellular vesicles or to the cell surface [3, 46]. Several lines of evidence for this interaction have been observed so far supporting this view, including a number of studies from our research group [8, 21, 22, 28]. The present work aimed to evaluate the role of the Cl^- -conducting proteins CFTR (cystic fibrosis transmembrane conductance regulator) and CIC-5 in modulating H^+ -ATPase in renal proximal tubule.

To our knowledge, the present paper is one of the

first studies of H^+ -ATPase and Cl^- interaction by means of *in vivo* microperfusion technique in kidney proximal tubule, although studies in other nephron segments have been reported [28]. In this series of experiments, we have observed that H^+ -ATPase-mediated bicarbonate reabsorption rate is reduced in the presence of the chloride channel inhibitor NPPB. Considering that the major fraction of proximal tubule HCO_3^- reabsorption is mediated by the apical membrane Na^+/H^+ exchanger NHE3 [47], these experiments were performed in the presence of the specific NHE3 inhibitor S3226. In fact, previous *in vivo* microperfusion studies indicated that under physiological conditions, V-type H^+ -ATPase is responsible for only 20 to 30% of total bicarbonate reabsorption in proximal tubule [48] and that in the absence of NHE3, no other EIPA-sensitive NHE isoform contributes to HCO_3^- reabsorption in this nephron segment. Thus, our data confirm those obtained by Malnic & Geibel in isolated rabbit S3 proximal tubules, in which H^+ -ATPase activity was also significantly reduced in the presence of NPPB [8].

To further establish a cell model to investigate the role of different Cl^- carrying conducting proteins on modulating of H^+ -ATPase activity, we used an immortalized rat renal proximal tubule cell lineage (IRPTC). As reported earlier, the main mechanisms of H^+ extrusion in these cells are the Na^+/H^+ exchanger NHE1 and H^+ -ATPase [36]. After inhibition of Na^+/H^+ exchanger-mediated H^+ extrusion by using a Na^+ -free solution, we have observed a significant reduction of H^+ -ATPase activity when alternatively NPPB or Cl^- -free solution were used, confirming previous *in vitro* studies [8, 49] that demonstrate a role for chloride in modulating H^+ -ATPase activity.

The role of chloride channels in H^+ -ATPase function has not only been explained by the establishment or dissipation of a potential difference across cellular and intracellular membranes. Some evidence has supported an additional, non electrical coupling of H^+ -ATPase and chloride. Kaunitz et al. [25] have shown that acidification of microsomes from rat renal medulla depends on chloride channels; however, even after dissipating the potential difference across endosome membranes with valinomycin, the ATPase still depends on the presence of Cl^- ions [25]. According to the authors, addition of valinomycin with equimolar potassium to these cells increased J_{H^+} by only a fraction of the transport rate observed with saturating chloride, suggesting that this transport was not a simple uniport, but instead a symport mechanism (H/Cl). Their model includes a transmembrane

segment with H⁺ and Cl⁻ conductances associated with a potential sensitive halide activator site [25]. In our model, even after abolishing the cell membrane PD by using valinomycin in a potassium-rich medium, apical V-ATPase activity was still dependent on chloride, suggesting that not only an ion shunt is responsible for the role of chloride on H⁺-ATPase function.

The mechanisms responsible for the differences in the requirement for chloride in the different tissues have not been well defined. Besides, there is still some controversy in relation to the role of different, associated Cl⁻-conducting proteins, including CIC family members and the cystic fibrosis transmembrane conductance regulator (CFTR) in modulating vacuolar H⁺-ATPase activity. A number of evidences have demonstrated that CFTR is a cAMP-regulated Cl⁻ channel. Phosphorylation by cAMP-dependent kinase activates the channel activity by reducing the channel-closed probability [50]. Indeed, we observed a significant increase in H⁺-ATPase activity in the presence of a membrane-permeant form of cAMP (data not shown), which could suggest that stimulation of chloride currents through cAMP-sensitive chloride channels may affect H⁺-ATPase activity. However, on the basis of these experiments, we cannot exclude a direct role (independent of CFTR) of cAMP in modulating H⁺-ATPase activity. In fact, studies by Brown & colleagues on A-type intercalated cells from kidney collecting duct have consistently shown that application of cAMP results in an increase of apical V-ATPase [51].

In the human kidney, CFTR channels are expressed in proximal tubules, thin limbs of Henle's loop, distal tubules, and collecting ducts [33]. In addition to the localization of CFTR in the apical surface of proximal tubules, subcellular distribution analysis indicates that the bulk of CFTR is found in endosomal fractions, where it co-distributes with CIC-5 and the V-ATPase. These findings indicate that besides playing a role in regulating chloride permeability across the apical membrane, CFTR is also involved with apical endocytosis [52]. In fact, Barasch and colleagues have demonstrated a deficient acidification of intracellular organelles in cystic fibrosis, indicating a possible role for CFTR in the organellar acidification dependent on V-type H⁺-ATPase [34]. Additionally, it was consistently shown in both *Cftr*^{-/-} mice and in patients with cystic fibrosis that the functional loss of CFTR leads to low molecular weight (LMW) proteinuria, again supporting a role of CFTR in proximal tubule endocytosis [52]. The data presented in the current work suggests a role of CFTR in modulating H⁺-ATPase

activity in plasma membrane of proximal tubule, since CFTR knockdown ultimately leads to a significant reduction of H⁺-ATPase function. Unexpectedly, the functional loss of CFTR was also found to be associated with a decrease of B2 subunit-V-ATPase expression. Although the determination of the factors involved in downregulation of B2 expression cannot be established on the basis of our present results, it is plausible to presume an indirect role of CFTR in modulating the synthesis or degradation of H⁺-ATPases, or even of one or more of its interacting partners.

It is widely known that CFTR is not the only chloride channel playing a significant role in H⁺-ATPase activity in endosomes and brush border membrane from proximal tubules. In this tubule segment, the co-localization of V-ATPases with CIC-5 chloride-proton exchanger in apical endosomes has been demonstrated [30]. Mutations in the gene *CLCN5* have been shown to decrease or abolish CIC-5 activity. This gene is related with Dent's disease, an X-chromosome-linked disease characterized by LMW proteinuria, hypercalciuria and kidney stones, a finding that has highlighted the importance of this channel in proximal tubule function [31, 53]. Indeed, recent studies have suggested that CIC-5 acts as a voltage-dependent electrogenic chloride/proton exchanger, which is thought to provide a chloride current to neutralize the increase of H⁺ in the intraorganellar space, permitting an effective acidification process [54-56]. Moreover, *in vitro* acidification of cortical renal endosomes prepared from CIC-5 knockout mice was shown to be significantly reduced. Further, the loss of CIC-5 function was found to reduce apical fluid-phase and receptor-mediated endocytosis, as well as the endocytotic retrieval of plasma membrane proteins [57]. All these findings support the importance of CIC-5 in endosomal acidification, essential for protein reabsorption.

The alterations observed in Dent's disease are not restricted to endosomal function. By analyzing renal biopsy specimens from patients with Dent's disease, Moulin and colleagues consistently noted inversion of H⁺-ATPase polarity in the proximal tubules to a basolateral distribution, in contrast to its apical localization in the normal kidney. The authors suggest a possible physical interaction between the C-terminus of CIC-5 and H⁺-ATPase, which could be essential for appropriate targeting or even stability of the multimeric complex [58]. On the other hand, in CIC-5 knockout mice models, it was consistently shown that the distribution and polarity of H⁺-ATPase remains apparently unaltered, maybe reflecting some species-related differences [59, 60].

In our cell model, derived from rat kidney proximal tubule, although changes in the expression of a number of proteins could be expected as a consequence of the immortalization process, ClC-5 knockdown ultimately led to a significant reduction of the H⁺-ATPase B2 subunit, possibly reflecting a role of ClC-5 in modulating its co-localized components or even its partners. In our experiments, the chloride conductance inhibition either by the use of NPPB itself or by using CFTR or ClC-5-siRNA based silencing ultimately did not alter H⁺-ATPase B2 subunit surface expression or total megalin expression, respectively. This could suggest that reduced H⁺-ATPase activity observed after inhibition of chloride conductances are not dependent on changes in the protein trafficking. Differently to the fast effects observed through the microperfusion studies, which can be related to changes of chloride conductance, the slow effects observed after CFTR or ClC-5 silencing reflect more profound

alterations on H⁺-ATPase, possibly indicating a role of these channels in modulating effectors of the signaling pathways involved with proton ATPase activity.

In conclusion, our observations point to a potential role of both CFTR and ClC-5 in modulating H⁺-ATPase function in plasma membrane of kidney proximal tubule.

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