Uroguanylin inhibits H+-ATPase activity and surface expression in renal distal tubules by a PKG-dependent pathway

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Submitted 23 December 2013; accepted in final form 13 July 2014

GUANYLINS COMPOSE A FAMILY of peptides that play an important role in the regulation of salt balance, pH regulation, appetite, and gut health (28). Among these peptides, uroguanylin (UGN) is the one with more pronounced renal actions (8).

In the kidney, UGN modulates the excretion of sodium, potassium, bicarbonate, chloride, and water (1, 8, 21, 29). These actions occur by altered tubular reabsorption and tubular secretion of these electrolytes along the nephron without changes in glomerular filtration rate. Besides, most of these effects are related to increase of cGMP levels in urine (8, 11).

In a previous study, our group has demonstrated that UGN inhibits bicarbonate reabsorption in proximal and distal tubules (1). In proximal tubules, the UGN effect was attributed to inhibition of Na+/H+ exchanger 3 (NHE3) activity by activation of both PKG and PKA pathways (21). However, in distal tubules, an additional mechanism, other than inhibition of the NHE, was suggested for UGN action on bicarbonate reabsorption (1).

The vacuolar H+-ATPase (V-ATPase) is the major cellular mechanism of H+ secretion/bicarbonate reabsorption in the α-intercalated cells of the distal segments of the nephron, playing an important role in lumen acidification (38). The V-ATPase is a large multisubunit protein that mediates ATP-driven vectorial H+ transport across cell membranes. Its activity is controlled by a number of different mechanisms, including regulation of the assembly of the V-ATPase via complex or dynamic regulation of its subunit expression in the cell membrane surface (10).

Among the multiple subunits, B1 seems to occupy a prominent role in the regulation of H+-ATPase. B1 subunit knockout mice develop tubular acidosis, metabolic acidosis, dehydration, and growth retardation (7). In addition, the inactivation of the B1 subunit in intercalated cells leads to type 1 distal renal tubular acidosis (dRTA), a disease associated with salt- and potassium-wasting nephropathy (14).

Considering that H+-ATPase in distal segments is constantly submitted to hormonal regulation, such as the renin-angiotensin-aldosterone system and natriuretic peptides (25, 26, 40), and that we have previously demonstrated that UGN inhibits hydrogen secretion in renal distal tubule, the purpose of the present study was to test the hypothesis that the UGN inhibitory effect on distal bicarbonate reabsorption might be dependent at least in part on H+-ATPase inhibition. The signaling mechanisms involved in the inhibitory effect of UGN on hydrogen secretion in renal distal tubules were also addressed. The findings of the present study show that UGN inhibits H+-ATPase activity in both rat renal distal tubule and in Madin-Darby canine kidney (MDCK)-C11 cells. This effect seems to be dependent on a reduction of the surface expression of B1 H+-ATPase subunit. In addition, the current data show that the cGMP/PKG signaling pathway and not cAMP/PKA is involved in the UGN regulation of distal bicarbonate reabsorption.

MATERIALS AND METHODS

Reagents and antibodies. All chemicals were obtained from Sigma Chemical (St. Louis, MO) unless otherwise noted. UGN was pur-

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chased from Bachem (Philadelphia, PA). KT5823, a specific inhibitor of PKG was purchased from Calbiochem (San Diego, CA). EZ-Link Sulfo-NHS-SS-Biotin as well as Immunopure immobilized streptavidin were purchased from Thermo Fisher Scientific (Rockford, IL). A monoclonal antibody (mAb) to H−-ATPase subunit B1 (V-ATPase B1) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mAb to actin (JLA20) was purchased from Merck Millipore (Billerica, MA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Life Technologies (Carlsbad, CA).

Animals. Animal procedures and protocols were followed in accordance with the ethical principles in animal research of the Brazilian College of Animal Experimentation and were approved by the Committee of the State University of Ceará and São Paulo. Experiments were performed using male Wistar rats (250–300 g) housed under standardized conditions (constant temperature of 22°C, 12-h dark-light cycle, and relative humidity of 60%) at the Superior Institute of Biomedical Sciences of State University of Ceará and of São Paulo. To perform stationary in vivo microperfusion, the animals were anesthetized with intramuscular ketamine (75 mg/kg) and xylazine (8 mg/kg). The left jugular vein was cannulated for infusion of 3% bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Cells. MDCK-C11 cells were obtained from Dr. Hans Oberleitner and used from passages (80−90). Serial cultures were maintained in DMEM low glucose supplemented with 5% l-glutamine, 5% sodium piruvate, 10% (vol/vol) heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were grown at 37°C, 95% humidified air-5% CO2 (pH 7.4) in a CO2 incubator (Lab-Line Instruments, Melrose Park, IL). The cells were subcultured with trypsin-EGTA (0.02%) and seeded on tissue culture plates containing sterile glass coverslips (for pH measurements) to become confluent. For all experiments, cells were plated in serum-free medium 24 h before the experiments.

Gene expression of guanylate cyclase-C receptor and MDCK-C11 cells. RNA was extracted from MDCK-C11 cells using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. cDNA synthesis from total RNA (1 μg) was produced by reverse transcription using the superscript III kit according to the manufacturer’s protocol (Invitrogen). PCR was performed using Taq-polymerase manufacturing protocol (Promega, Madison, WI). Briefly, thermal cycling for initial guanylate cyclase-C (GC-C) analysis included a denaturation step at 95°C for 5 min followed by 36 cycles of 95°C for 30 s, annealing temperature of 64.5°C for 30 s and 72°C for 1 min. 28S gene was used as a housekeeping gene. The initial 28S analysis included a denaturation step at 95°C for 5 min followed by 36 cycles of 95°C for 30 s, annealing temperature of 60°C for 30 s and 72°C for 1 min. Primer sequences and expected product lengths were as follows: GC-C receptor (XM_543798.2) primer: forward 5’- AAC-CATTGGCGATGCCTACA-3’ and reverse 5’- AGTTGGCGAGCATGTGCAAA-3’, 491 bp; and 28S primer: forward 5’- TCATCGAGCCCAAAAGG-3’ and reverse 5’- GATTCGGCAGGTGAGTT-3’, 487 bp. PCR products were resolved by electrophoresis at 100 V through 2% agarose gel and visualized with GelRed (Biotium, Hayward, CA).

Fluorescence microscopy. Intracellular pH was measured spectrophotometrically at 37°C with the fluorescent pH-sensitive probe 2’7’-bis (2-carboxyethyl)-5(6)-carboxyfluorescein acetoxyethyl ester (BCECF-AM). Cells grown to confluence on glass coverslips were loaded with the dye by exposure for 5 min to 12 μM BCECF-AM in the control solution (Table 1). The acetoxyethyl ester form of BCECF enters the cell and is rapidly converted to the anionic-free acid form by intracellular esterases. Following the loading period, the glass coverslips were rinsed with the control solution to remove the BCECF-containing solution and placed in a thermoregulated chamber mounted on an inverted epifluorescence microscope (Nikon, Tokyo, Japan). The measured area under the microscope had a diameter of 260 μm and contained on the order of 40 cells. Bathing solutions were rapidly exchanged without disturbing the position of the coverslips. Fluorescence was monitored using 440 (pH insensitive) or 495 nm (pH sensitive) alternately as excitation wavelengths, using a xenon light source. Emission was measured at 530 nm by a photomultiplier-based fluorescence system, at time intervals of 5 s. pH 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 (Table 1), at pH 6, 7, and 8 (36).

Cell pH recovery. Cell pH recovery was evaluated following the acidification of pH with the NH4Cl pulse technique (2) after a 2-min exposure to 20 mM NH4Cl (Table 1), in the absence of external Na+

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Table 1. Composition of solutions

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Control</th>
<th>NH4Cl</th>
<th>0 Na+</th>
<th>Calibration</th>
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<tbody>
<tr>
<td>NaCl</td>
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<td>121.0</td>
<td>—</td>
<td>20.0</td>
</tr>
<tr>
<td>KCl</td>
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<td>5.4</td>
<td>5.4</td>
<td>130.0</td>
</tr>
<tr>
<td>CaCl2</td>
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<td>1.0</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>—</td>
</tr>
<tr>
<td>MgCl2</td>
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<td>0.5</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
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<td>0.4</td>
<td>0.4</td>
<td>—</td>
</tr>
<tr>
<td>Na2HPO4</td>
<td>0.3</td>
<td>0.3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>HEPES</td>
<td>10.0</td>
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<td>10.0</td>
<td>5</td>
</tr>
<tr>
<td>Glucose</td>
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<td>0.6</td>
<td>0.6</td>
<td>—</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>—</td>
<td>20.0</td>
<td>—</td>
<td>—</td>
</tr>
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<td>7.4</td>
<td>7.4</td>
<td>6.5 / 7.0 / 7.5</td>
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<tr>
<td>Nigericin</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.00267</td>
</tr>
</tbody>
</table>

All values are in millimolars, except for pH; HCl or NaOH were used in all Na+-containing solutions to titrate to the appropriate pH, and KOH was used in the Na+-free solution. HEPES, 4-2-hydroxyethyl-1-piperazineethanesulfonic acid; NMDG, N-methyl-D-glucamine.

(Table 1), to inhibit the NHE, with or without several inhibitors, as described later. In all the experiments, we calculated the initial rate of pH recovery (dpHi/dt, pH U/min) from the first 2 min after the start of the pH recovery curve by linear regression analysis.

cGMP and cAMP assay. MDCK-C11 cells were cultured to 100% confluence in 96-well plates. Cells were incubated for 10 min with culture medium containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) or IBMX plus 10^{-6} M UGN or 10^{-6} M atrial natriuretic peptide (ANP) (used as positive control in cGMP assay) or 10^{-4} M forskolin (used as positive control in cAMP assay). cGMP and cAMP were measured by using the Amersham Enzyme immunoassay Bio-trak (EIA) System (GE Healthcare, Piscataway, NJ) according to specifications of the manufacturer.

Determination of PKA activity in cell lysates. MDCK-C11 grown to confluence in 24-well plates were treated or not with UGN and subsequently solubilized in lysis buffer containing 20 mM MOPS, 50 mM β-glycerol phosphate, 50 mM NaF, 1 mM sodium vanadate, 5 mM EGTA, 2 mM EDTA, 1% NP40, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. PKA activity was measured in this cell lysate using a nonradioactive PKA Kinase Activity Assay (Enzo Life Sciences, Farmingdale, NY), according to the manufacturer’s instructions.

Determination of PKG activity in cell lysates. MDCK-C11 grown to confluence in 24-well plates were treated or not with UGN and subsequently solubilized in extraction buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 1 μg/ml pepstatin, 0.5 μg/ml leupeptin, 2 mM NaF, 0.2 mM Na3VO4, and 5 mM β-mercaptoethanol. PKG activity was measured in this cell lysate using a single-site and semiquantitative CycLex cGK Assay Kit (CycLex, Nagano, Japan) according to the manufacturer’s instructions.

Cell surface biotinylation. The assay was performed as described previously (3). Cells were rinsed twice in ice-cold PBS-Ca-Mg (PBS with 0.1 mM CaCl2, 1.0 mM MgCl2). Surface membrane proteins were then biotinylated by incubating the cells twice for 25 min with 2 ml of ice-cold biotinylation buffer (150 mM NaCl, 10 mM triethanolamine, 2 mM CaCl2, and 1.5 mg/mL EZ-Link sulfo-NHS-SB-biotin). Cells were then rinsed twice for 20 min with a quenching solution alone or together with UGN (10^{-6} M) and/or HMA (10^{-4} M), a selective inhibitor of NHE. B: tubules were perfused with 8br-cAMP (10^{-6} M) and/or HMA (10^{-4} M). Numbers of perfused tubules are indicated in the bars. Data are means ± SE. *P < 0.05 vs. control.
buffer (PBS-Ca-Mg, 100 mM glycine), washed twice with ice-cold PBS-Ca-Mg, and strapped into ice-cold solubilization buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% sodium deoxycholate, and 1% Triton X-100, pH 7.4) containing protease inhibitors (0.7 μg/ml pepstatin A, 0.5 μg/ml leupeptin, and 40 μg/mL PMSF). After lysis on ice for 60 min, extracts were centrifuged for 10 min at 14,000 g and 4°C. The protein concentration of the supernatants was measured according to Lowry et al. (22), and equal protein amounts of cell lysate (500 μg) were equilibrated with streptavidin-agarose beads at 4°C. Before the addition of streptavidin, an aliquot of the supernatant was saved for analysis of total B1 subunit H-ATPase protein expression by immunoblotting. The beads were then washed three times in ice-cold solubilization buffer. Biotinylated proteins were released by incubation in Laemmli buffer and subjected to SDS-PAGE and immunoblotting.

SDS-PAGE and immunoblotting. Protein samples were solubilized in Laemmli sample buffer and separated by SDS-PAGE using 7.5% polyacrylamide gels. For immunoblotting, proteins were transferred to PVDF (Millipore Immobilon-P; Millipore, Bedford, MA) at 350 mA for 8–10 h at 4°C with a TE 62 transfer electrophoresis unit (GE Healthcare). Sheets of PVDF containing transferred proteins were incubated first in Blotto (5% nonfat dry milk and 0.1% Tween 20 in PBS, pH 7.4) for 1 h to block nonspecific binding of antibody, followed by overnight incubation in primary antibody diluted in Blotto (1:1,000). The sheets were then washed in Blotto and incubated for 1 h with an appropriate HRP-conjugated secondary antibody diluted 1:2,000 in Blotto. After being washed five times in Blotto and two times in PBS (pH 7.4), the sheets were incubated in an enhanced chemiluminescence reagent for 1 min and then placed in a digital imaging system (ImageQuant LAS 4000 mini, GE HealthCare) to

Fig. 3. The inhibitory effect of UGN on H⁺ secretion in rat renal distal tubule is mediated by PKG. A: experiments were performed in the presence or absence of UGN (10⁻⁶ M) and KT5823 (10⁻⁶ M), a selective inhibitor of PKG, which prevented the effect promoted by the peptide. B: tubules were perfused with control solution with or without uroguanylin and H89 (10⁻⁷ M), a PKA inhibitor, which was unable to prevent the inhibitory effect of uroguanylin. Numbers of perfused tubules are indicated in the bars. Data are means ± SE. *P < 0.05 vs. CTRL; &P < 0.05 vs. KT5823 + UGN.

Fig. 4. Original traces of fluorescence microscopy experiments with Madin-Darby canine kidney (MDCK)-C11 cells. The records show the recovery of pHi along 2 min after an acid pulse with NH₄Cl. The figure shows 3 different conditions. A: control solution with 135 mM Na⁺. B: 0 Na⁺ solution. C: 1 μM UGN added to the 0 Na⁺ solution.
visualize the bands. The quantification was realized using ImageJ densitometry software.

Statistical analysis. The data were analyzed by a Visual Basic program in Excel software. Statistical comparisons were made by the unpaired $t$-test, taking the probability of 0.05 (5%) as the limit of significance. When more than two groups were compared, one-way ANOVA followed by Tukey’s post hoc test, taking 0.05 (5%) as the limit of significance, was performed. In microperfusion experiments, a minimum of six tubules was used ($n$ = number of perfused tubules).

RESULTS

UGN inhibits $H^+$-ATPase-mediated proton secretion in the rat distal tubule. We have previously demonstrated that UGN inhibits distal bicarbonate reabsorption (1) in the presence or absence of HMA, an NHE inhibitor. These previous results suggest that UGN inhibits NHE-dependent and $H^+$-ATPase dependent proton secretion in this nephron segment. To test the hypothesis that UGN inhibits $H^+$-ATPase activity in the distal nephron, we performed in vivo stationary microperfusion experiments in which we perfused distal segments of the nephron with UGN alone or together with CONC, an $H^+$-ATPase inhibitor, and/or HMA. The perfusion of CONC together with HMA significantly inhibited the secretion of $H^+$ in rat distal tubules compared with the control solution (Fig. 1). Addition of UGN to CONC + HMA also caused a significant inhibition compared with the control, and this inhibition was similar to the one found when we perfused the distal tubules with HMA + CONC (Fig. 1). This finding demonstrates that the effect of UGN on distal $H^+$ secretion involves inhibition of no mechanism other than NHE or $H^+$-ATPase.

![Graph A](image1.png)
![Graph B](image2.png)

Fig. 5. Uroguanylin inhibits Na$^+$-independent pH recovery and hydrogen flux ($J_H$) in MDCK C11 cells. MDCK-C11 cells were treated with control solution (135 mM Na), 0 Na solution, or 1 μM uroguanylin diluted in 0 Na solution. After intracellular acidification by means of the ammonium pulse technique in MDCK-C11 cells, the initial rates of pH recovery (pH U/min) were calculated from the curves by linear regression analysis (A). B: plotted rates of hydrogen extrusion, which were obtained from the product of $dpHi/dt$ and intracellular buffer capacity $\beta_i$ of the average pH of the experimental groups ($pHi \approx 6.5$). UGN significantly inhibits Na$^+$-independent pH recovery and hydrogen extrusion in MDCK-C11. Number of experiments is indicated in the bars. *$P < 0.05$ vs. control, #P < 0.05 vs. 0 Na.

![Graph A](image3.png)
![Graph B](image4.png)

Fig. 6. Uroguanylin inhibits $H^+$-ATPase-dependent pH recovery in MDCK-C11 cells by a PKG-dependent pathway. MDCK-C11 cells were treated with 1 μM UGN or 8br-cGMP in the presence of EIPA (NHE1 inhibitor) and Schering (SCH, H/K-ATPase inhibitor), and/or KT5823 (KT, PKG inhibitor) diluted in 0 Na solution, after intracellular acidification by means of the ammonium pulse technique. The initial rates of pH recovery (pH U/min) were calculated from the curves by linear regression analysis (A). B: plotted rates of hydrogen extrusion, which was obtained from the product of $dpHi/dt$ and intracellular buffer capacity $\beta_i$ of the average pH of the experimental groups ($pHi \approx 6.5$). UGN significantly inhibits $H^+$-ATPase-dependent pH recovery and hydrogen extrusion in MDCK-C11 cells. Number of experiments is indicated in the bars. *$P < 0.05$ vs. EIPA + SCH.
Signaling mechanisms mediating the effects of UGN on bicarbonate reabsorption in rat renal distal segments. Considering that the guanylate cyclase/cGMP/PKG pathway has been described as the classical signaling mechanism for guanylin actions (8), we then examined whether the activation of this signaling pathway mediates the effect of UGN in the distal tubule. We first evaluated the effect of a cGMP analog, 8-bromoguanosine (8Br)-cGMP, on distal bicarbonate reabsorption. As shown in Fig. 2A, the perfusion of distal segments with 1 μM 8Br-cGMP significantly inhibited the distal hydrogen secretion similarly to UGN. Besides, in the presence of HMA (NHE inhibitor), there was a slight tendency of an additional inhibition of hydrogen secretion by 8Br-cGMP, compared with HMA alone, but it was not significant, probably attributable to the reduced permeability of the cGMP analog (11). Nevertheless, as illustrated in Fig. 3A, there was no significant change in bicarbonate reabsorption when the distal segments were perfused with UGN and the PKG inhibitor, KT5823, compared with the control or to the group perfused only with KT5823. However, these groups were significantly different from the group perfused only with UGN (Fig. 3A). These results indicate that PKG inhibition prevents the UGN inhibitory effect on hydrogen secretion in rat distal tubules, suggesting that the inhibition of bicarbonate reabsorption by UGN is mediated, at least in part, by activation of the cGMP/PKG signaling pathway.

In a previous study, we showed that the UGN inhibitory effect on proximal bicarbonate reabsorption was dependent on both PKG and PKA activation (21). Thus we evaluated the possible involvement of PKA on UGN action in distal tubule. As seen in Fig. 2B, the cAMP analog, 8Br-cAMP, produced a tendency to inhibit hydrogen secretion although this trend did not reach statistical significance. Besides, as illustrated in Fig. 3B, the PKA inhibitor, H89, associated with UGN was unable to reverse the inhibitory effect of this peptide on distal hydrogen secretion, suggesting that PKA activation is not involved in the UGN distal effect.

UGN inhibits H⁺-ATPase-dependent pHi recovery in MDCK-C11 cells. With the purpose to further investigate the inhibitory effect of UGN on H⁺-ATPase activity, we performed fluorescence microscopy experiments to investigate the UGN effect on H⁺-ATPase-dependent intracellular pH recovery in the distal tubule cell line, MDCK-C11, a cell line with H⁺-secretory α-intercalated cell characteristics (12). In Fig. 4, we can observe the original representative records of pHi recovery experiments after an acid pulse with NH₄Cl. As depicted in the figure, there was an important inhibition of the rate of pHi recovery in 0 Na⁺ solution (Fig. 4B) compared with the original record of an experiment in the presence of Na⁺ (Fig. 4A). Under incubation with 1 μM UGN, the inhibition of the Na⁺-independent pHi recovery was even more pronounced (Fig. 4C). In Fig. 5A, the mean values of the pHi recovery rates and hydrogen flux of the groups discussed above are plotted. The hydrogen flux (JH⁺) was calculated from the following
equation: 

\[ J_{\text{H}^+} = \frac{d\text{pHi}}{dt} \beta_i \]

where \( d\text{pHi}/dt \) is the rate of pHi recovery after the acid load and \( \beta_i \) is the cytosolic buffering capacity averaged for the respective pH range. We compared the rates of \( H^+ \) extrusion at the average pH of all experimental groups, and the respective average \( \beta_i \) of MDCK-C11 cells was used according to Fernandez et al. (6). The mean pHi used in the calculation of H fluxes corresponds to mean values obtained during the pHi recovery period after the ammonium pulse. These data show the significant inhibition of Na\(^+\)-independent pH recovery rates promoted by UGN (Fig. 5, A and B).

MDCK-C11 cells express three mechanisms related to intracellular pH regulation. Three mechanisms related to intracellular pH regulation as expressed by MDCK-C11 cells are NHE1 (isoform 1 of the NHE), \( H^+/K^+ \)-ATPase, and \( H^+ \)-ATPase (6, 12). Therefore, to further investigate whether the effect of UGN on Na\(^+\)-independent pH recovery in MDCK-C11 cells involved the inhibition of \( H^+ \)-ATPase, fluorescence microscopy experiments were performed in the presence of 0 Na\(^+\) solution, to inhibit Na\(^+\)-dependent pHi recovery; experiments with EIPA, an NHE1 inhibitor, and Schering, an \( H^+/K^+ \)-ATPase inhibitor, were also performed. In these conditions, incubation of UGN promoted a significant additional inhibition of pHi recovery and hydrogen flux, compared with the group treated with the inhibitors only. These data indicate that UGN inhibits the pHi recovery and H\(^+\) flux dependent on \( H^+ \)-ATPase activity (Fig. 6, A and B).

To investigate the involvement of cGMP/PKG pathway in this inhibitory effect of UGN, we observed the effect of a cGMP analog, 8Br-cGMP, on \( H^+ \)-ATPase-dependent pHi recovery. Incubation of the cGMP analog caused a significant inhibition of \( H^+ \)-ATPase activity, in a similar magnitude as did UGN (Fig. 5). Moreover, administration of KT5823, a PKG inhibitor, prevented the effect promoted by UGN (Fig. 6), demonstrating that inhibition of \( H^+ \)-ATPase by UGN involves PKG activation.

**Effect of UGN on intracellular contents of cGMP and cAMP, PKG and PKA activities, and GC-C expression in MDCK-C11 cells.** Considering the possible involvement of the cGMP/PKG pathway in the inhibitory effect of UGN on \( H^+ \)-ATPase activity (Figs. 3A and 6), we evaluated the effect of UGN on the intracellular cGMP concentration and PKG activity in distal tubule cells. Figure 7A illustrates that the incubation of MDCK-C11 cells with UGN for 10 min promoted a significant increase of intracellular cGMP content. In this experiment, ANP, an agonist of GC-A, was used as a positive control, which demonstrated an effect similar to UGN. Furthermore, both UGN and ANP were also able to stimulate a significant increase of PKG activity in MDCK-C11 cells (Fig. 7B). Considering these findings, we next examined and confirmed that MDCK-C11 cells express the classical guanylin receptor, GC-C (Fig. 7C). It is worth mentioning that the GC-C mRNA could only be detected when higher amounts (give the used concentration) of cDNA template were used for amplification (Fig. 7C), suggesting that MDCK cells display very low endogenous expression of this receptor.

We also evaluated the effect of UGN on intracellular cAMP concentration and PKA activity. Incubation of MDCK-C11 cells with UGN did not change either intracellular cAMP content or PKA activity (Fig. 8, A and B). In these experiments, forskolin was used as a positive control, and, in contrast to the UGN effect, forskolin incubation promoted a significant increase of both intracellular cAMP concentration and PKA activity.

**Effect of UGN on \( H^+ \)-ATPase surface expression in distal tubule cells.** To investigate the mechanism by which UGN inhibits \( H^+ \)-ATPase activity, we next examined whether UGN was capable of decreasing the B1 \( H^+ \)-ATPase subunit cell surface expression in MDCK-C11 cells (Fig. 9). The total levels of B1 \( H^+ \)-ATPase subunit (Fig. 9A), the \( \beta \) subunit of \( H^+/K^+ \)-ATPase (Fig. 9B), and actin in 15-min vehicle, UGN, and UGN + KT5823-treated cells were examined to ensure that the yield of protein extracted was constant in each of the
These findings reinforce our previous study, which suggested that no additional inhibitory effect was promoted by UGN. The diuretic, natriuretic, and kaliuretic properties of UGN are now recognized. In addition, a potential effect of this peptide on renal tubular acidification has been demonstrated (1, 21). In the kidney, it has been demonstrated that pendrin, an anion exchanger found in intercalated cells, is downregulated at the transcriptional level by UGN (29). Recently, our group has also demonstrated a role for UGN in the control of luminal acidification (1, 21). Our present findings show that UGN inhibits H\(^{+}/K\)\(^{-}\)-ATPase-mediated hydrogen secretion, in the presence of HMA, involves inhibition of H\(^{+}/ATPase\) (1).

MDCK-C11 cells have properties of intercalated cells, which secrete protons and chloride in the collecting duct of mammals (6, 12). Because we have proposed to evaluate H\(^{+}/ATPase\) activity, our ph\(\text{II}\) recovery experiments were undertaken in the presence of NHE1 and H/K-ATPase inhibitors because these transporters are also constitutively expressed in MDCK-C11 cells (6). Thus, in the presence of their respective inhibitors and 0 Na\(^{+}\) solution, the remaining hydrogen secretion in MDCK-C11 cells would be performed by H\(^{+}/ATPase\). In these conditions, our findings clearly showed that UGN inhibits the Na\(^{+}\)- independent ph\(\text{II}\) recovery exerted by H\(^{+}/ATPase\).

Several studies indicate an important role for guanylin in pancreatic and intestinal secretion of HCO\(_{3}^{-}\) (17, 18, 19, 32). In intestinal cells, guanylin, uroguanylin, and the thermo-stable toxin of Escherichia coli (Sta) stimulate anion secretion into the intestinal lumen. This mechanism involves the increase of intracellular cGMP by activating a GC-C. The activation of this pathway leads to phosphorylation of the cystic fibrosis transmembrane regulator (CFTR) by PKG (18, 32). Activation of CFTR stimulates the activity of a Cl/HCO\(_{3}^{-}\) exchanger, which promotes an increase in secretion of HCO\(_{3}^{-}\) into the intestinal lumen (19).

In the kidney, it has been demonstrated that pendrin, an anion exchanger found in \(\beta\)-intercalated cells, is downregulated at the transcriptional level by UGN (29). Recently, our group has also demonstrated a role for UGN in the control of luminal acidification (1, 21). Our findings suggest that guanylin peptides inhibit bicarbonate reabsorption in proximal and distal experimental conditions. As observed in the representative immunoblotting shown in Fig. 9, A and C, the inhibition of H\(^{+}/ATPase\) by UGN was accompanied by a reduction of the surface amount of V-ATPase. The peptide induced an inhibition of surface H\(^{+}/ATPase\) \(\beta\) subunit by 54 \(\pm\) 8\%, relative to control. In addition, in the presence of KT5823, a PKG inhibitor, this effect of UGN was abolished. Consistent with the functional findings shown in Figs. 1 and 5, UGN did not affect the cell surface expression of the \(\beta\) subunit of H\(^{+}/K\)\(^{-}\)-ATPase in MDCK-C11 cells (Fig. 9, B and D), suggesting that this peptide does not modulate H/K-ATPase-mediated hydrogen secretion in the distal tubule.

DISCUSSION

The diuretic, natriuretic, and kaliuretic properties of UGN are now recognized. In addition, a potential effect of this peptide on renal tubular acidification has been demonstrated (1, 21, 29). In a previous study, we demonstrated that UGN inhibits bicarbonate reabsorption/hydrogen secretion in rat renal proximal and distal segments (1). Moreover, an additional mechanism, besides inhibition of NHE2, was proposed for the effect of UGN on hydrogen secretion in distal segments because, in the presence of the NHE inhibitor HMA, UGN promoted an additional inhibitory effect on hydrogen secretion (1).

Our present findings show that UGN inhibits H\(^{+}/ATPase\) activity but not H\(^{+}/K\)\(^{-}\)-ATPase-mediated hydrogen secretion in the distal nephron cells, both in vivo and in vitro. In micropерfusion experiments conducted in the presence of the Na\(^{+}/H\)\(^{+}\) exchanger (HMA) and H\(^{+}/ATPase\) (CONC) inhibitors, no additional inhibitory effect was promoted by UGN. These findings reinforce our previous study, which suggested that the remaining inhibitory effect promoted by UGN on distal bicarbonate reabsorption, in the presence of HMA, involves inhibition of H\(^{+}/ATPase\) (1).

Fig. 9. UGN decreases the surface expression of H\(^{+}/ATPase\) \(\beta\) subunit in MDCK-C11 cells by a PKG-dependent mechanism. A and B: equivalent quantities (50 \(\mu\)g) of total cellular lysates from MDCK-C11 cells treated for 15 min with vehicle (CTRL), UGN, or UGN combined with the PKG inhibitor were subjected to SDS-PAGE and immunoblotting. A: representative immunoblotting using the monoclonal antibody against the \(\beta\) subunit of H\(^{+}/K\)\(^{-}\)-ATPase. Actin was used as an internal control. B: representative immunoblotting using the monoclonal antibody against the \(\beta\) subunit of H\(^{+}/K\)\(^{-}\)-ATPase. Actin was used as an internal control.

C and D: cell surface biotinylated proteins, after treatment with UGN or UGN + KT5823 (PKG inhibitor) for 15 min, were subjected to SDS-PAGE and immunoblotting. Immunoblot analyses were performed using a monoclonal antibody against the \(\beta\) subunit of H\(^{+}/K\)\(^{-}\)-ATPase (C) or a monoclonal antibody against the \(\beta\) subunit of H\(^{+}/K\)\(^{-}\)-ATPase (D). The amount of H\(^{+}/ATPase\) \(\beta\) subunit and H\(^{+}/K\)\(^{-}\)-ATPase \(\beta\) subunit were quantitated by densitometry. Number of experiments are indicated within the parentheses. **P < 0.01 vs. CTRL.
segments of the nephron (1, 20, 21). In contrast, recently, Rozcenfeld and coworkers (29) have demonstrated that UGN downregulates the pendrin gene in β-intercalated cells, which in turn would inhibit bicarbonate secretion. It is possible that this effect of UGN in β-intercalated cells could be sort of a compensatory mechanism for the peptide inhibitory effect on distal hydrogen secretion through α-intercalated cells of UGN.

The inhibition of H+-ATPase by a cGMP-dependent mechanism has been described in plants (35) and in rat cortical collecting ducts (37). In the current study, we demonstrated that a permeable cGMP analog inhibits distal hydrogen secretion. In addition, a previous study from our laboratory (20) has also demonstrated the inhibition of distal hydrogen secretion by renoguanin, a new member of the guanylin family, through a mechanism dependent on PKG activation. Moreover, the expression of GC-C receptor has been demonstrated in rat renal cortex (21) and, also, in MDCK-C11 cells, favoring the activation of the classical guanylin receptor by UGN in these studies. In fact, our data show that UGN incubation was able to stimulate the increment of the intracellular level of cGMP and PKG activity in MDCK-C11 cells, which indicates the activation of the classical pathway by UGN in this study.

In addition to the undeniable importance of the GC-C pathway on guanylin action, especially during salt overload, when this receptor is upregulated (9, 27), the presence of an additional receptor, other than GC-C, for UGN effect in the kidney has been suggested (33). The mentioned studies have shown that GC-C knockout mice still exhibit UGN-induced natriuresis (5) and also that UGN could activate a G protein receptor sensitive to pertussis toxin, in proximal tubule cells (34). Moreover, we have previously demonstrated that UGN inhibits bicarbonate reabsorption in proximal tubules through inhibition of NHE3 activity by a mechanism dependent on the activation of, not only PKG, but also the PKA pathway. However, this same study showed that the activation of adenylyl cyclase was not involved in the proximal effect of UGN (21).

In contrast to our previous work in proximal tubules (21), the present observations showed that the PKA pathway was not involved in the distal effect of UGN on hydrogen secretion because the inhibition of PKA did not prevent the inhibitory effect of UGN on distal hydrogen secretion. Besides, neither the intracellular content of cAMP nor PKA activity were changed by UGN incubation of MDCK-C11. Accordingly, the perfusion of distal segments with a cAMP analog did not promote a significant inhibitory effect of distal hydrogen secretion.

The activity of H+-ATPase may be regulated by trafficking, domain assembly/disassembly, and changes in the ratio of ATP hydrolysis/H+ pumping, as well as by other means (39). Subcellular localization, regulation, and functional differences of H+-ATPase populations may at least in part be regulated by the presence of specific subunit isoforms. The B1, α4, and δ2 isoforms have been labeled as intercalated cell specific (38). Holliday et al. (15) reported that the amino-terminal domains of both isoforms of the B subunit, B1 and B2, contain binding sites to F-actin, which may be responsible for the interaction between V-ATPase and actin filaments in vivo and could allow for the observed trafficking (15).

The importance of the B1 subunit for the regulation of the proton pump has been evidenced in patients with the inherited form of type 1 dRTA. In humans, mutations of the gene encoding for the B1 subunit (Atp6v1b1) in intercalated cells of distal tubule have been involved in the pathogenesis of this disease (16). The characteristics of dRTA are not limited to abnormal acid-base balance; hence it causes metabolic acidosis but often includes a salt- and potassium-losing nephropathy that may lead to hypokalemia and dehydration (14, 30, 31). In the current study, we demonstrate that the inhibitory effect of UGN on H+-ATPase activity in distal tubule involves reduction of H+-ATPase B1 subunit abundance in the plasma membrane of distal tubule cells. Besides, this effect was abolished in the presence of the PKG inhibitor, reinforcing the involvement of the GC-C/cGMP/PKG pathway in the distal effect of UGN.

In summary, our findings indicate a role for UGN, a peptide known for its effect in the regulation of sodium homeostasis, also in acid-base balance, by inhibiting distal bicarbonate reabsorption. We suggest that UGN activates GC-C receptors in distal segments of the nephron by increasing intracellular cGMP content, leading to PKG activation and decreasing insertion of H-ATPase B1 subunit in the apical membrane of α-intercalated cells. The data presented herewith also corroborate with previous studies that have postulated a role for intercalated cells in the maintenance of body fluid and electrolyte balance, involving inhibition of the B1 subunit of H+-ATPase (14). Furthermore, our current and previous findings (21) and those of others (23, 26, 40) suggest UGN as a possible counterregulator for the actions of the renin-angiotensin-aldosterone system, a well-known promoter of sodium retention and acid extrusion by the kidneys.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

GRANTS
This work was supported by the funding agencies FAPESP, CNPq, and FUNCAP.

AUTHOR CONTRIBUTIONS

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