Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2011;28:733-742

Accepted: August 26, 2011

Uroguanylin Regulates Net Fluid Secretion via the NHE2 Isoform of the Na^+/H^+ Exchanger in an Intestinal Cellular Model

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Key Words

Water transport • Intracellular pH • Uroguanylin • T84 cells • Na^+/H^+ exchanger • Sildenafil

Abstract

Uroguanylin (UGN) has been proposed as a key regulator of salt and water intestinal transport. Uroguanylin activates cell-surface guanylate cyclase C receptor (GC-C) and modulates cellular function via cyclic GMP (cGMP), thus increasing electrolyte and net water secretion. It has been suggested that the action of UGN could involve the Na^+/H^+ exchanger, but the actual contribution of this transporter still remains unclear. The objective of our study was to investigate the putative effects of UGN on some members of the Na^+/H^+ exchanger family (NHEs), as well as to clarify its consequences on transepithelial fluid flow in T84 cells. In order to do so, transepithelial fluid flow (J_V) was studied by optic techniques and intracellular pH (pH_i) was measured with a fluorescence method. Results showed that NHE2 is found at the apical membrane and has a major role in Na^+ absorption; NHE1 and NHE4 are localized at the basolateral membrane with a house-keeping role in steady state pH_i. In the assayed conditions, cell exposure to apical UGN increases net secretory J_V, without changing short-circuit currents nor transepithelial resistance, and reduces NHE2 activity. Therefore, at physiological pH, the effect on net J_V was produced mainly by a reduction in normal Na^+ absorption through NHE2, rather than by the stimulation of electrolyte secretion. Our study shows that the effect of UGN on pH_i is GC-C/cGMP-mediated and enhanced by sildenafil, thus involving PDE5 enzyme. Additionally, cell exposure to apical UGN results in intracellular alkalinization, probably due to indirect effects on basolateral NHE1 and NHE4, which have a major role in pH_i regulation.

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Introduction

Regulation of salt and water intestinal transport is crucial in maintaining body fluid volume. This process is mediated *in vivo* by the combined action of many hormones, neurotransmitters and local factors. The guanylin family has been suggested as a critical contributor in this activity [1-3]. This family is a group of small

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1015-8987/11/0284-0733$38.00/0

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cysteine-rich endogenous peptides with close structural similarities with the *Escherichia coli* heat-stable enterotoxin (STa). In a similar way as STa, these peptides activate cell-surface guanylate cyclase C receptors (GC-C), thus modulating cellular function via the intracellular second messenger, cyclic GMP (cGMP) [4-6]. Functional *in vitro* and *in vivo* studies on GC-C agonist effects, have described the participation of the guanylin-peptides/GC-C system on the regulation of 1) the fluidity of intestinal content, by stimulating Cl^- secretion and inhibiting NaCl absorption, with the following decrease in water absorption, which results in an increase of net fluid secretion [7]; 2) the pH of intestinal content, by inhibiting H^+ extrusion through the Na^+/H^+ exchanger, and 3) Na^+ homeostasis, especially in cases of high salt intake, by limiting intestinal Na^+ uptake and stimulating Na^+ kidney excretion [8]. One of the well-known members of this family is Uroguanylin (UGN), which is produced by the intestinal mucosa and released to the extracellular media on both luminal and serosal sides. This peptide has shown to have effects on salt and water transport in both intestine and kidney [9-11].

Although it is well accepted that rises in intracellular cGMP levels in intestinal cells elicit an increase in electrolyte and water secretion, through stimulation of Cl^- secretion and inhibition of Na^+ absorption, this modulation appears to vary with the agonist involved. STa acts as a GC-C super-agonist, producing large and uncontrolled increases in cGMP, which results in secretory diarrhea [12]. On the other hand, UGN stimulation of GC-C and increase in Cl^- secretion seems to depend on mucosal acidity, attaining higher intracellular cGMP levels and electrogenic Cl^- secretion with lower pH values [13]. In addition, some authors have suggested that the mechanism of action of this compound could also involve the Na^+/H^+ exchanger (NHE). Indeed, this exchanger plays a crucial role in electrically neutral Na^+ absorption in mammalian intestine [14]. However, the contribution of NHE to UGN action has not been fully demonstrated [15, 16].

The human colonic carcinoma-derived T84 cell line expresses high levels of GC-C and has thus been used as an intestinal model to study this receptor and the cGMP-mediated signaling pathway [17]. Although T84 cell line has classically been used as a cellular model for Cl^- secretion under stimulated conditions [18], their Na^+ absorption capacity has been reported and informed as HOE-694 sensitive, indicating the participation of the Na^+/H^+ exchanger [19]. Additionally, their high-transepithelial resistance minimizes paracellular fluid flux, facilitating the study of fluid movement associated to transcellular processes. Our previous studies demonstrated that in T84 cells STa induces significant and strong net water secretion, even in the absence of osmotic or chemical gradients, in part coupled to an electrogenic Cl^- secretion and in part linked to non-electrogenic ionic transfer [20]. We have also demonstrated that Na^+/H^+ exchanger is the main transporter involved in pH regulation in these cells [21]. Together, these features led us to hypothesize that in T84 cells the net fluid secretion, associated to the non-electrogenic ionic transfer, would be related to a decrease in Na^+ absorption through the Na^+/H^+ exchanger. Therefore, the aim of this study was to investigate the putative effects of UGN on some members of the Na^+/H^+ exchanger family (NHEs), as well as to clarify its consequences on transepitelial fluid flow in T84 cells. Although NHE1, NHE2 and NHE4 expression in T84 cells has been reported, their cellular localization and function have not been well established [22]. For this reason, in the present work we first evaluated the functional localization of the different NHE isoforms in T84 polarized cells.

Our results showed that NHE2 is expressed at the apical membrane while NHE1 and NHE4 are expressed at the basolateral membrane. NHE1 and NHE4 were found to play a house-keeping role in maintaining the steady state of intracellular pH. Apical NHE2 was inhibited by UGN through the GC-C/cGMP pathway, involving the 5-phosphodiesterase enzyme (PDE5), and leading to an increase in net secretory fluid flow. This is the first time that transepithelial fluid flow induced by UGN is measured directly.

Materials and Methods

Cell culture

Serial cultures of T84 cells (American Type Culture Collection, Rockville, MD, USA; passages 46–51) were grown as monolayers in a flask with 1:1 v/v mixture of DMEM and Ham's F12 medium (Invitrogen, San Diego, CA, USA) supplemented with 14 mM $NaHCO_3$, 15 mM Na-HEPES, 10 U/ml penicillin-streptomycin, 3.2 mM glutamine and 5% fetal bovine serum (Internegocios S.A., Mercedes, Bs. As., Argentina), adjusted to a pH of 7.4, in a 5% CO_2 atmosphere at 37°C.

After confluence, cells were routinely passed once a week, using trypsin. All experiments were performed on monolayers at passages 62-76, that were grown on permeable supports (Transwells, 3 μM pore size, 4.5 cm^2 growth area, Corning-Costar, NY, USA) during 18 to 22 days. This permeable support allows independent and free access to both apical and basolateral baths.

Primers	Sequences	Amplified fragment size (bp)	cDNA sequence location (nt)	Data Bank accession number
NH1F	5′- GACTACACACACGTGCGCACCCC -3′	233	860-882	GI 27777631
NH1R	5′-TCCAGGATGATGGGCGGCAGCAGGAAGAGGAA -3′		1092-1061	
NH2F	5′- GAAGATGTTTGTGGACATTGGGG -3′	550	1685-1707	GI 38569454
NH2R	5′- CGTCTGAGCTGCTGCTATTGC -3′		2234-2214	
NH3F	5′- AGAAGCGGAGAAACAGCAG -3′	217	2070-2088	GI 6806920
NH3R	5′- GGAGAAAACACAGGGTTGTC -3′		2286-2267	
NH4F	5′-AAGAATATCCGCTACCTCTCC TA -3′	192	2231-2254	GI 141802649
NH4R	5′-CTGTGTAGGCTCTTCATTGGTAT -3′		2423-2401	

Table 1. Specific primers used to detect the expression of NHE isoform.

RNA extraction and Reverse Transcriptase (RT)-PCR assay

Total RNA from human epithelial colonic cells (positive control, kindly provided by Dr. C. Ibarra) or from T84 cells, was isolated using the Trizol method (Invitrogen, CA, USA) according to manufacturer's instructions. Reverse transcription was performed with 2 µg of total RNA using the Omniscript RT kit (Qiagen, CA, USA) with 10 µM of random decameres (Amersham) as primers, 10 units of RNAse inhibitor (Amersham) and 4 units of Omniscript RT enzyme, in a final volume of 20 µl. Reactions were incubated at 37°C during 60 minutes.

cDNA was then amplified using specific primers for human NHE1, NHE2, NHE3 and NHE4 isoforms. Sequences of the synthetic oligonucleotides, targets and expected product sizes are given in Table 1. A positive control for RNA integrity was included in each experiment using β-actin-specific primers (sense: 5' CGG AAC CGC TCA TTG CC 3'; antisense: 5' ACC CAC ACT GTG CCC ATC TA 3'). All reactions were carried out using 1 unit of Taq Recombinant DNA polymerase (Invitrogen, CA, USA) and 0.4 µM of specific primers in the presence of 1.5 mM $MgCl_2$ in a final volume of 25 µl. Cycling steps (repeated 35 times) were preceded by a denaturation step of 120 s at 94ºC and were followed by an extension step of 10 min at 72ºC. The cycling profile involved: 30 s at 94ºC, 30 s at 55ºC and 60 s at 72ºC for NHE1 and NHE4, and 30 s at 94ºC, 30 s at 62ºC and 85 s at 72ºC for NHE2 and NHE3. The absence of DNA was confirmed by PCR of total RNA, instead of cDNA.

Intracellular pH studies

T84 monolayers were grown on permeable filters that were then inserted between two lucite frames diagonally placed in a quartz cuvette, thus separating two different fluid compartments. Free access to both apical and basolateral baths was therefore possible, as previously reported [23].

Measurements were made with a computerized and thermo regulated (37°C) spectrofluorometer (Jasco 770, Easton, MD, USA). Cell monolayers inserted in the frame were placed forming a 45° angle with the exploring beam. For pH_i measurements, the cells were loaded with 6 µM fluorescent probe 2′,7′bis(2-carboxyethyl)- 5, 6- carboxyfluorescein acetoxymethyl ester (BCECF-AM) in control solution in both apical and basolateral compartments, for 30 min at 37°C. Fluorescence emission was monitored at 535 nm, with excitation wavelengths of 439 and 510 nm. The ratio of the BCECF fluorescence emitted from dye-loaded cells was calibrated, in terms of pH, by incubating the cells with "high K^+ solution" (in mM: 140 KCl, 4.6 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 5 glucose) and then by permeabilizing the cells with 5 µM nigericin to balance extracellular pH (pH_o) with intracellular pH (pH_i). Then the pH-bathing solution was stepped between pH 6.5 and 8. The 510/439 ratio was linear over this pH range ($r = 0.99$, $n = 6$).

In some experiments pH_i regulation was investigated by monitoring pH_i recovery after an acid load by the NH_4Cl-prepulse technique, as previously described [24].

Measurements of fluid fluxes

Confluent T84 cells grown on filters were directly inserted between two barrel-shaped lucite hemi-chambers, so as to define two independent compartments, as previously described [25]. One of the compartments (basolateral) was open to the atmosphere, whereas the other compartment (apical) was hermetically sealed. A positive hydrostatic pressure gradient (4.5 cm H_2O) was continuously applied to the apical bath. The closed chamber was connected with a small-diameter polyethylene tube to the net water measurement system, where the net transepithelial fluid flow (J_V) was recorded every minute, as previously reported [26]. Briefly, the position of a liquid meniscus inside a capillary tube was photo-electrically detected. Displacements to the right or to the left were proportional to the amount of water moving across the tissue layer. The system's sensitivity was 50 nl. The data were computed in microliters per minute per square centimeter. The basolateral bath was continuously bubbled with the appropriate CO_2-O_2 mixture to maintain the pH of the medium at 7.4 ± 0.1 (37°C).

In order to evaluate the UGN effect on the apical side of the monolayer and after 10 min in control condition, the apical side of the chamber was open. The register of UGN conditions started 5 minutes after system stabilization.

Electrophysiological studies

Transepithelial resistance (R_T) was measured every two days with a Millicell-ERS electric resistance system (Millipore) in cells grown on permeable supports (Transwells, 3 µM pore size, 4.5 cm^2 growth area, Corning-Costar, NY, USA). These measurements were performed in cells exposed to the culture medium (DMEM –F12). During the experiments, short-circuit current (I_{SC}) was continuously recorded with an automatic voltage-clamp system (Physiological Instruments) and

Navycite (ME2AG4) electrodes. R_T was estimated, during the experiments, every 90 s from current deflections in response to a 1 $mV.s^{-1}$ pulse.

Solutions and chemicals

In functional studies, cells were bathed on each side with a control saline solution (Control) containing, in mM: 141 NaCl, 5.4 KCl, 1.0 $CaCl_2$, 0.4 KH_2PO_4, 0.5 $MgCl_2$, 0.4 $MgSO_4$, 0.3 Na_2HPO_4, 10 Hepes, 6 glucose. Ammonium solution (NH_4^+) was obtained by replacement of 20 mM NaCl with NH_4Cl. Na^+ free solution (0Na) was achieved by equimolar replacement of sodium with N-methyl D-glucamine. All solutions were titrated to pH 7.4 (at 37°C). Solution osmolalities (296 ± 4 $mOsm.kg_{H2O}^{-1}$) were routinely measured in a pressure vapor osmometer (Vapro; Wescor, Logan, Utah, USA). When specified, the inhibitors ethyl-isopropyl-amiloride (EIPA) and HOE-694 (a benzoylguanidine derivative) were used. BCECF-AM was purchased from Molecular Probes (Eugene, OR). HOE-694 was kindly provided by Dr. Juergen Puenter (Sanofi-Aventis Deutschland GmbH, Frankfurt, Germany). Sildenafil citrate was obtained from Pfizer. 8Br-cGMP, DB-cAMP and all other substances were obtained from Sigma.

Data analysis and statistics

Results are expressed as mean ± SEM. Student's t test for paired or unpaired data, as appropriate, was used. $p < 0.05$ was considered statistically significant.

Results

Na^+/H^+ exchanger isoform expression and functional localization in T84 cells

It has been reported that T84 cells express three isoforms of the Na^+/H^+ exchanger (NHE1, NHE2 and NHE4) [22]. However, there is also evidence that in some intestinal models, culture conditions (*e.g.*, time after seeding or cellular density) may be decisive in determining the expression of NHE isoforms [27]. Therefore, before conducting functional experiments, we first assessed NHE expression by RT-PCR at three different post-seeding times (days 10, 15 and 22). In all cases T84 cells formed confluent monolayers. Maximal transepithelial resistance (R_T = (2181 ± 86) $\Omega.cm^2$; n=40) was reached around the 17th day, without presenting significant additional changes up to the 22nd day. RT-PCR experiments were performed using specific primers for human NHE isoforms (Table 1) [22, 28, 29] and β-actin as an internal control. Assays were undertaken using total RNA extracted from T84 cells and from human epithelial colonic cells (human intestine, used as positive control). Figure 1A shows the results of a representative RT-PCR experiment using total RNA from cells on the 22nd post-seeding day. We found positive bands of ~233 bp for NHE1, ~550 bp for NHE2 and ~192 bp for NHE4 in both intestine and T84 cells. Additionally and as expected, a proper-sized fragment for NHE3 (~ 217 bp) was obtained from the RT-PCR of human intestine RNA, but not from T84 cells, regardless of the post-seeding time. These results confirm that NHE1, NHE2 and NHE4 -but not NHE3- mRNA is present in T84 cells, and also propose that isoform expression is independent of the level of confluence or differentiation of T84 cells.

We then investigated the functional localization of each NHE isoform. In order to do so, pH_i measurements were carried out in T84 cells grown on permeable filters, allowing independent and free access to both cell baths. All experiments were performed in HCO_3^--free solutions, to minimize the effect of bicarbonate-transporting systems found in T84 cells [21]. Cells were first incubated in a solution containing Na^+ (Control solution). In these conditions, steady state pH_i was 7.48 ± 0.02 (n=112). Apical and basolateral Na^+/H^+ exchange activity was assessed by monitoring Na^+-dependent pH_i recovery after cellular acidification by the classical NH_4Cl prepulse technique [24], followed by the bilateral removal of Na^+ (0Na solution) and the subsequent restoration of Na^+ to the basolateral, the apical or both compartments (Fig. 1B). As shown in Figure 1C, no significant pH_i recovery was observed when Na^+ was removed from both basolateral and apical baths or from the basolateral bath alone. In contrast, Na^+ removal from only the apical bath allowed a slight pH_i recovery, nevertheless attaining a final pH_i that was significantly lower than the observed in control conditions. To further investigate the putative participation of different Na^+/H^+ exchanger isoforms in these responses, the rate of pH_i recovery was evaluated in the presence of apical or basolateral specific NHE inhibitors. Figure 1D illustrates the results obtained using 100 μM EIPA, a derivative of amiloride that at this concentration inhibits all isoforms of the Na^+/H^+ exchanger [30], and the selective inhibitor HOE-694, that at 1 μM inhibits the NHE1 isoform, while at 10 μM inhibits both NHE1 and NHE2 isoforms [23]. Regardless of the bath, EIPA significantly decreased pH_i recovery rate; however, this effect was much more important when added to the basolateral bath. Additionally, pH_i recovery was significantly reduced by the apical addition of HOE-694, only at doses necessary to block NHE2 (10 μM). In contrast, pH_i recovery was abolished by the addition of even 1 μM HOE-694 to the basolateral bath and higher doses did not elicit further inhibition.

Altogether, these functional results suggest that both NHE1 and NHE2 participate in pH_i recovery after an

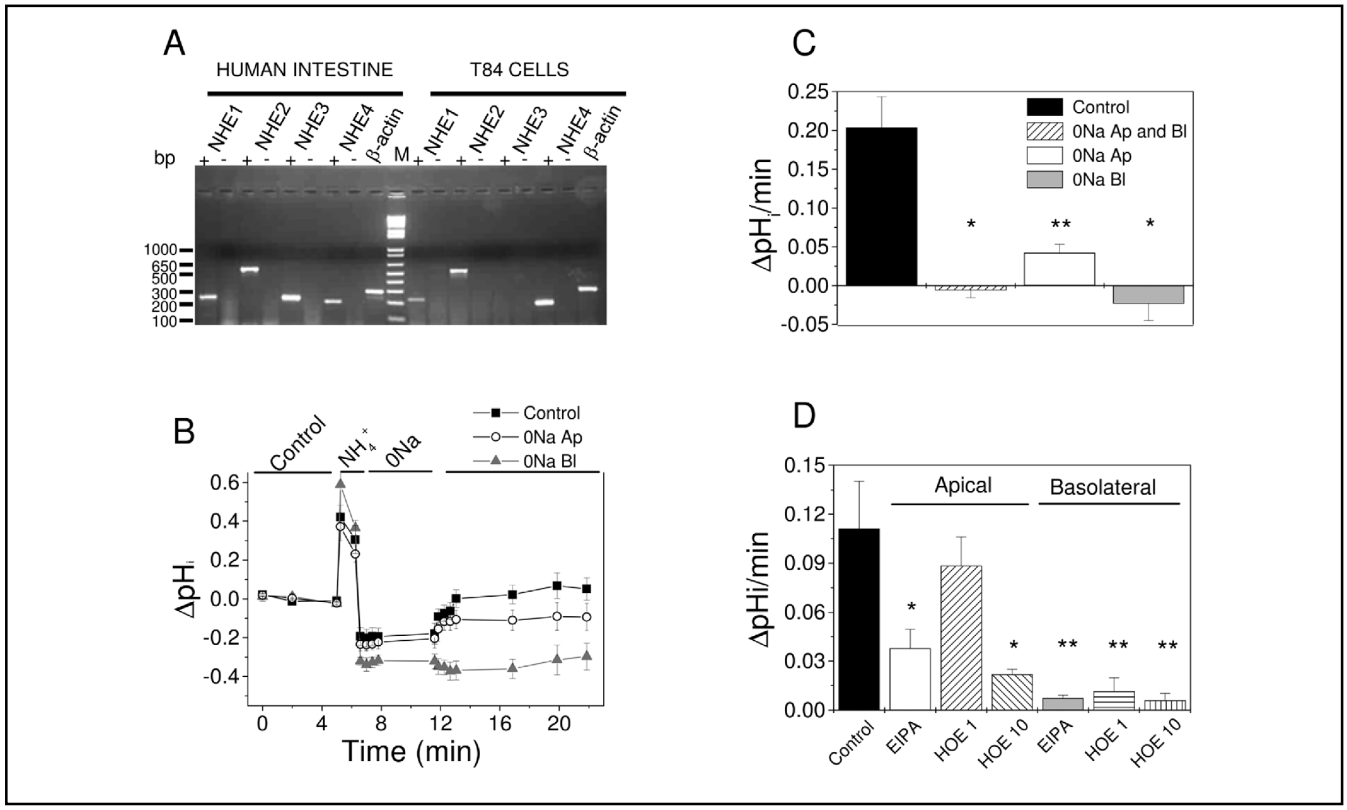

Fig. 1. Molecular and functional identification of NHE isoforms in T84 cells. A- RT-PCR experiment using specific primers for human NHE1, 2, 3 and 4 isoforms and mRNA obtained from human intestinal epithelial cells (positive control) and T84 cells. An aliquot (10 µl) of each reaction was subjected to electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. Assays were carried out in the presence of cDNA (+) or RNA (-) as templates. Central lane (M): 1Kb Plus DNA marker. β-actin was used as an internal control. The experiment shown is representative of at least four other assays from cells on the 22nd post-seeding day. B- Time course of pH_i regulation after an acid load. Cells were exposed, in both baths, for 1 min to a solution containing NH_4Cl (NH_4^+), then to a solution without Na^+ (0Na). Subsequently Na^+ was restored to the basolateral (○, 0Na Ap), the apical (▲ , 0Na Bl) or both (■, control) compartments. When only the apical or the basolateral side of the monolayer was exposed to 0Na, the opposite side was filled with control solution. C- Initial rate of pH_i recovery in all the conditions described above. Values are mean ± SE of 4 to 8 independent experiments. *$p < 0.001$ ** $p < 0.01$ vs control. D- Initial rate of pH_i recovery after an acid load, in the presence of 100 µM EIPA (EIPA), 1 µM HOE-694 (HOE 1) or 10 µM HOE-694 (HOE 10) to determine the relative participation of NHE1 and NHE2 isoforms. The pharmacological agents (EIPA and HOE-694) were solubilized in control solution at experimental concentrations and added to the indicated compartment, whereas the opposite compartment was filled with control solution. Values are mean ± SE of 3 to 5 independent experiments. *$p<0.05$; **$p< 0.02$ vs control.

acid load in T84 cells, but their localization and degree of contribution are quite different. In fact while NHE1 is found on the basolateral side and appears to have a significant influence on pH_i recovery, NHE2 is found on the apical side and seems to play a smaller role. Finally, NHE4 appears to not be involved in this response.

Effects of Uroguanylin on water flux, electrical parameters and Na^+/H^+ exchanger activity in T84 cells

To evaluate the putative participation of NHEs in fluid movement across T84 cells stimulated by GC-C agonists, we investigated the effect of UGN on net transepithelial fluid flow (J_V), on electrical parameters (R_T and I_{SC}) and on Na^+/H^+ exchange activity. Cells were first incubated in a HCO_3^--free solution at a pH of 7.4 (Control solution) and all parameters were evaluated under this condition and after the addition of 0.1 µM UGN to the apical bath (a concentration previously shown to provoke significant intracellular cGMP accumulation in T84 cells [31]). Figure 2A shows that under control conditions, T84 cells have a basal net fluid secretion that increases significantly in the presence of apical UGN. This apical UGN-induced J_V reached its maximum after 10 min of UGN exposure and persisted throughout the entire experiment (30 min). The mean values of maximum J_V in both experimental conditions are shown in Figure 2A insert. Interestingly, exposure to apical

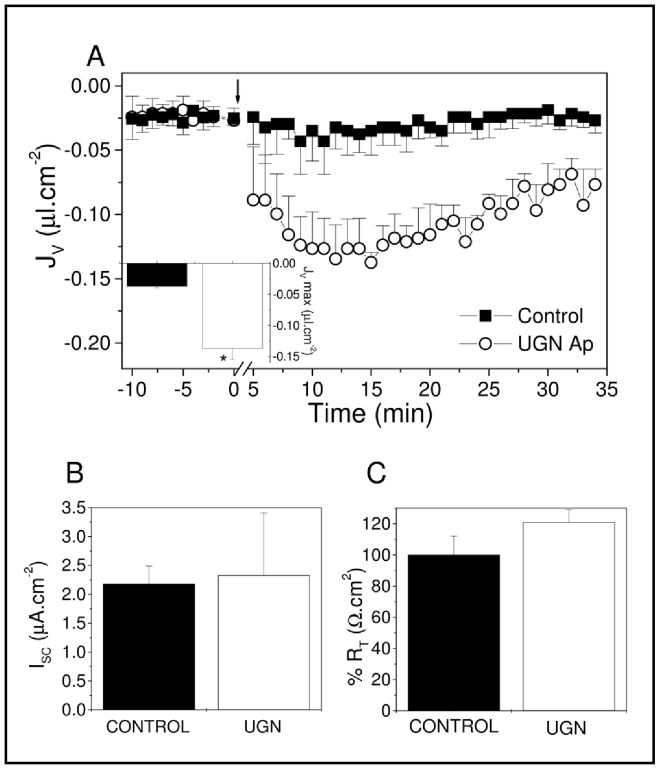

Fig. 2. Effects of UGN on J_V, I_{SC} and R_T in T84 cells. A- Time course of J_V under control conditions (■, control, pH=7.4) or in the presence of apical 0.1 µM UGN (○, UGN Ap) added at the arrow. Curves are mean ± SE of 4 independent experiments. The insert shows the mean values of maximum J_V reached at the described conditions. Negative values indicate secretory flux. B and C- Simultaneous evaluation of the transepithelial resistance (R_T) and short-circuit current (I_{SC}) in both assayed conditions. No significant differences were observed indicating the conservation of epithelial barrier functions and a non-electrogenic phenomenon. * p<0.01 UGN vs control.

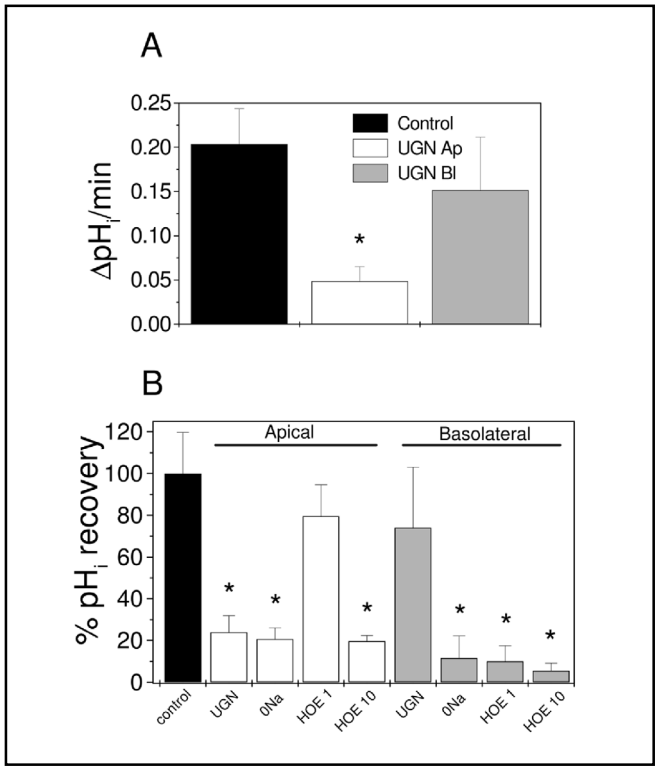

Fig. 3. Effect of UGN on Na^+/H^+ activity in T84 cells. A- Initial rate of pH_i recovery after an acid load (NH_4^+) under apical 0.1 µM UGN (UGN Ap), basolateral 0.1 µM UGN (UGN Bl) and Control conditions. Values are mean ± SE of 5 to 9 independent experiments, *p < 0.01 UGN vs control. B- Comparison of the percentage of pH_i recovery rate with apical or basolateral UGN (UGN), 10 µM HOE-694 (HOE 10), or Na^+-free solution (0Na). It is noticed that UGN effect is similar to that of specific inhibitors and of sodium absence, only when acting on the apical side of the cell. *p<0.01 vs control.

UGN during 30 min did not affect R_T or I_{SC} (Figs. 2B and C). These results strongly suggest that -in the absence of chemical or osmotic gradients, and at physiological pH- UGN-induced net secretory transepithelial fluid flow is associated to an electrically silent ion transport.

The next step was to investigate whether 0.1 µM UGN affects Na^+/H^+ activity. As shown in Figure 3A, after an acid pulse protocol, pH_i recovery significantly decreased in the presence of apical UGN. On the contrary, basolateral UGN did not affect this parameter. Even more, apical UGN-induced inhibition was ~80%, which is comparable to the percentage obtained by blocking apical NHE activity either by Na^+ removal or by the addition of 10 µM HOE-694 (Fig. 3B). These results strongly suggest that UGN has an inhibitory effect on the apical NHE2 isoform of the Na^+/H^+ exchanger.

Effects of Uroguanylin on steady state pH_i in T84 cells: role of Na^+/H^+ exchanger isoforms

We further investigated the action of UGN on apical NHE activity, evaluating its effects on steady state pH_i. The experimental conditions were the same as those in which UGN effects on J_V and on electrical parameters were studied. Unexpectedly, apical inhibition of NHE with UGN produced a small and transient increase in pH_i (Fig. 4A). Since it has been reported that UGN stimulation increases cGMP levels in T84 cells [31], and that PDE5 is a major cGMP-degrading phosphodiesterase in these cells [32, 33], the specificity of UGN effect was evaluated by using UGN plus sildenafil (a PDE5-specific inhibitor) and these effects were compared to those obtained with a permeable analogue of cGMP (8Br-cGMP). It can be noted that exposure to UGN plus sildenafil resulted in a stronger and more sustained UGN-induced intracellular

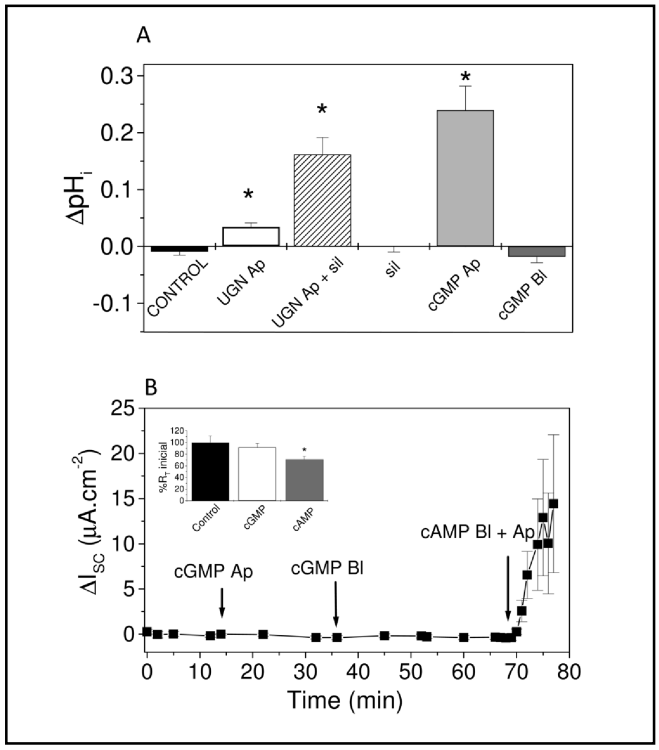

Fig. 4. Effect of UGN and cGMP on steady state pH_i in T84 cells. A- Steady state pH_i measured in basal conditions (control) and after apical exposure to 0.1 μM UGN (UGN Ap); UGN plus 1 μM sildenafil citrate (UGN Ap + sil); sildenafil alone (sil); 100 μM 8Br-cGMP -a permeable analogue of cGMP- in apical (cGMP Ap) or basolateral baths (cGMP Bl). ΔpH_i is the difference between initial pH_i (basal) and pH_i measured after solution change. Values are mean ± SE of 3 to 10 independent experiments. B- Time course of I_{SC} in the presence of 100 μM 8 Br-cGMP to apical (cGMP Ap) or basolateral (cGMP Bl) baths and 100 μM DB-cAMP to both apical and basolateral baths (cAMP Bl + Ap), added at the arrow. Insert shows the percentage of control R_T values measured in the assayed conditions. Values are mean ± SE of 5 independent experiments. *p<0.001 experimental vs control for 3A and B.

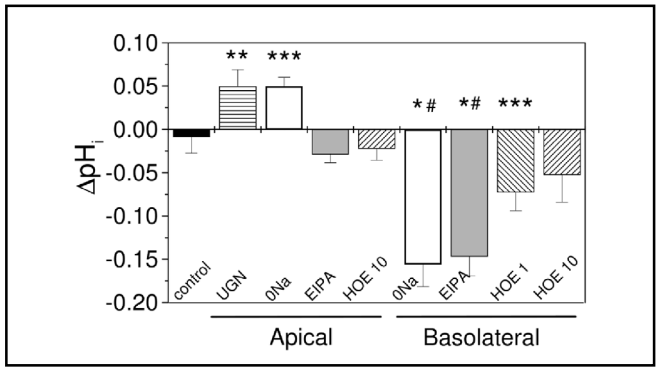

Fig. 5. Relative contribution of NHEs isoforms to steady state pH_i. pH_i was measured after changing apical or basolateral solutions and ΔpH_i is the difference between initial pH_i (basal) and pH_i measured after solution change. Pharmacological agents (100 μM EIPA, 10 μM HOE-694 and 0.1 μM UGN) were added in the indicated compartment, whereas the opposite compartment was filled with control solution. 0Na indicates Na^+-free solution in the specified compartment. Values are mean ± SE of 4 to 10 independent experiments, *p<0.001; **p<0.02; ***p<0.05 experimental vs control. # p<0.05 when comparing basolateral 0Na or 100 μM EIPA conditions vs basolateral 1 μM or 10 μM HOE-694 conditions.

alkalinization (Fig. 4A). Additionally, apical -but not basolateral- cGMP induced an intracellular alkalinization very similar to that evoked by UGN plus sildenafil (Fig. 4A). This cGMP response was not paralleled with changes in I_{SC} nor in R_T (Fig. 4B). This lack of effect on electrical parameters was not due to cellular unresponsiveness neither to loss of cellular integrity of the T84 monolayer, since a well described cAMP permeable analogue (DB-cAMP) clearly increased I_{SC} (Fig. 4C). Altogether, these results suggest that UGN specifically increases steady state pH_i via the activation of a cGMP-cascade that probably involves the PDE5 enzyme.

To explain this apical UGN-induced intracellular alkalinization, it was essential to evaluate the relative contribution of each of the three NHE isoforms to steady state pH_i. In order to do so, pH_i experiments were carried out under basal conditions, either in the absence of Na^+ or in the presence of blockers (HOE-694 or EIPA). Figure 5 shows that, when apical Na^+ was removed, a significant pH_i alkalinization was observed and, notably, its magnitude was comparable to that caused by UGN. However, in the presence of apical 100 μM EIPA or 10 μM HOE-694, no significant changes in basal pH_i were noticed. In contrast, basolateral Na^+ removal significantly decreased basal pH_i and this reduction was comparable to that obtained with 100 μM EIPA basolateral. 1 μM HOE-694 basolateral, also significantly reduced basal pH_i, whereas higher doses did not elicit further acidification. However, a significant difference was observed when comparing EIPA and HOE-694 basolateral effects. These results clearly indicate that another isoform, besides NHE1, is also expressed at the basolateral membrane, probably NHE4. Therefore, the intracellular alkalinization observed either in the absence of apical Na^+ or in the presence of apical UGN, could be due to an increase in the activity of basolateral exchangers in response to an enhanced Na^+-driving force. To confirm this hypothesis, we explored the putative participation of basolateral NHE isoforms in

the intracellular alkalinization, exposing T84 cells to the apical analogue of cGMP (8Br-cGMP) in the presence of basolateral NHE inhibitors. Figure 6 shows that cGMP-apical-induced alkalinization was abolished when basolateral isoforms were blocked with 1 µM HOE-694 or with EIPA. Altogether these results demonstrate that increases in pH_i induced by UGN or cGMP are due to the well coordinated activity of apical NHE2 and basolateral NHEs, via increases in sodium driving force.

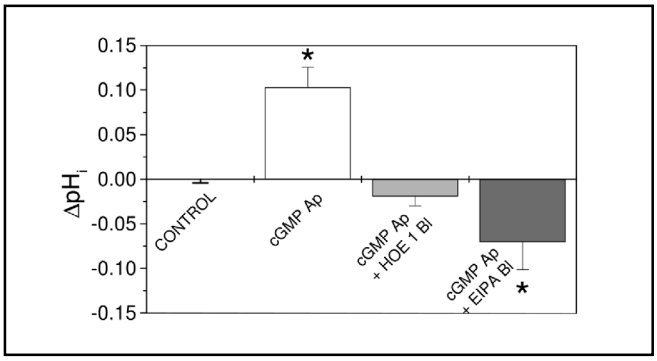

Fig. 6. Effect of simultaneous exposure to apical cGMP and basolateral NHE inhibitors on steady state pH_i in T84 cells. pH_i measured after T84 cells exposed to apical cGMP analogue, 8Br-cGMP, alone (cGMP Ap) and simultaneously with basolateral HOE-694 (cGMP Ap + HOE1 Bl) or EIPA (cGMP Ap + EIPA Bl). Values are mean ± SE of 3 to 5 independent experiments, *$p < 0.01$ experimental vs control.

Discussion

All guanylin peptides have shown to bind to the GC-C receptor, leading to an increase in Cl^- secretion and an inhibition of Na^+ absorption. However, the transporters involved in this process have not been well defined [16]. Considering previous results with STa (a GC-C super agonist), endogenous GC-C agonist UGN could shed some light on research in this direction.

Our results showed that, in T84 cells under physiologic conditions, net basal fluid secretion was significantly increased by apical UGN, without affecting R_T or I_{SC}. Thus, in the absence of chemical or osmotic gradients, UGN-induced net secretory transepithelial fluid flow is associated to an electrically silent ion transport. There is previous evidence that UGN produces an increase in Cl^- secretion under mucosal-acidic stimulation. However, transepithelial fluid flow was not measured under these conditions [13]. Even more, our previous studies in T84 cells under physiologic conditions proved that the effect of a GC-C super agonist (STa) on fluid flux has two components: an electrogenic effect (produced by Cl^- secretion) and a non-electrogenic effect [20]. The direct measurement of fluid flux simultaneous to a null I_{SC}, allowed us to identify, for the first time, the presence of the non-electrogenic component modulated by UGN.

Since NHE expression has been reported in T84 cells [21], this transporter is a strong candidate to explain this non-electrogenic component associated to fluid flux. Our results confirm that NHE1, NHE2 and NHE4 -but not NHE3- isoforms are expressed in T84 cells, but they are differentially localized: NHE1 and NHE4 were functionally found on the basolateral side, while NHE2 was shown on the apical side. Even more, our results indicate that while NHE1 and NHE4 would have a house-keeping role in steady state pH_i NHE2 would be mainly involved in Na^+ uptake. In fact, NHE2 has shown to be a

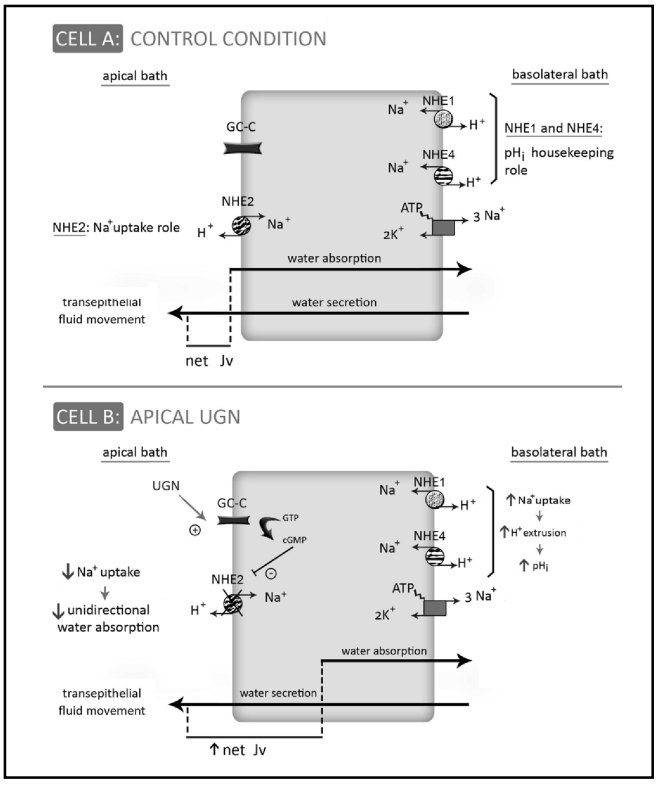

Fig. 7. Schematic model to explain the non-electrogenic component of UGN effects on NHEs activity and fluid secretion. Localizations of NHE isoforms, net fluid (J_V) and Na^+ fluxes on polarized T84 cells are schematically represented. Cell A: in basal conditions cells present net secretory J_V. Transcellular Na^+ movement is in mucosal-serosal direction, in part via apical NHE2 and basolateral Na^+/K^+/ATPase. Cell B: when cells are exposed to apical UGN, NHE2 is inhibited, thus reducing apical Na^+ uptake and consequently unidirectional water absorption. Hence, there is an increase in the Na^+ driving force between the extracellular basolateral compartment and the intracellular compartment. The basolateral NHEs increase their activity, alkalinizing the intracellular compartment.

major pathway for NaCl absorption in rat proximal colon [34] and to participate in Na^+ absorption in mouse colonic crypts [35]. Additionally, the capacity of T84 cells for absorbing Na^+ has been reported and informed as HOE-694 sensitive, indicating the participation of the Na^+/H^+ exchanger [19].

Our results also show that exposure of T84 cells to apical, but not basolateral, UGN produced a significant decrease on NHE activity, similar to that obtained with apical HOE-694 blockage and in absence of Na^+. This strongly suggests that UGN inhibits apical NHE2. This inhibition may properly explain UGN-induced net J_V secretion -towards the apical bath- through the UGN-induced reduction in Na^+ absortion. This UGN effect would decrease not only pH_i recovery but also the absortive component of J_V osmotically linked to NaCl uptake. Similar results regarding the Na^+-associated J_V were reported in renal cells [36].

Interestingly, under steady state conditions, apical UGN produced an intracellular alkalinization comparable, but lower, to that evoked by exposure to apical cGMP. Similar levels of alkalinization were obtained when UGN was added in the presence of sildenafil (protecting cGMP degradation). These results support the specificity of UGN response on steady state pH_i and confirm that PDE5 is the major phosphodiesterase involved in cGMP degradation in T84 cells as previously reported [32]. Moreover, under steady state conditions, the permeable analogue of cGMP was unable to increase I_{SC} in T84 cells. This result is in agreement with those published by Forte et al. [37], and confirms that the observed responses on J_V and pH_i are associated with a non-electrogenic process.

However, how could this alkalinization induced by UGN be interpreted and, in turn, what is its relationship with net J_V increase? Figure 7 shows the model proposed by us, additional and complementary to the well-established electrogenic effect of UGN on Cl^- secretion, to explain the non-electrogenic components of the effect of UGN. To understand these events, it was crucial to consider the relative contribution of each NHE isoform to either steady state pH_i or Na^+ absorption. Results showed that both basolateral isoforms (NHE1 and NHE4) are the main regulators of steady state pH_i in these cells, whereas the main role of the apical isoform (NHE2) is on sodium absorption. Our results show that the intracellular alkalinization observed in the presence of apical UGN could easily be explained by an increase in the activity of basolateral exchangers, in response to an enhanced Na^+ driving force. In steady state conditions, net Na^+ movement is in apical–basolateral direction: Na^+ enters the cell, at least in part, through apical NHE2, down its electrochemical gradient, and exits via the basolateral Na^+/K^+/ATPase pump (Fig. 7, cell A). Blockage of NHE2 (by UGN or cGMP) reduces apical Na^+ uptake. This 1) enhances Na^+ driving-force between the extracellular basolateral compartment and the intracellular compartment, activating NHE1 and/or NHE4, and thus increasing pH_i, and 2) reduces fluid flux associated to Na^+ absorption, thus increasing net secretory J_V (Fig. 7, cell B). To confirm this hypothesis, we explored the putative participation of basolateral NHE isoforms in the intracellular alkalinization, exposing T84 cells to apical cGMP in the presence of basolateral NHE inhibitors. Results showed that the intracellular alkalinization (produced by apical UGN or cGMP) depends on the activity of basolateral NHEs. Thus, the explanation model proposes that the increase in pH_i induced by UGN is due to the well coordinated activity of apical NHE2 and basolateral NHEs, via increases in Na^+ driving force. Additionally, these events affect transepithelial water movement.

In summary, our results show that the exposure of T84 cells to UGN increases net secretory fluid flux by inhibiting apical NHE2 isoform, which has a major role in Na^+ absorption. In the assayed conditions, this effect is mediated mainly by a reduction in normal Na^+ absorption through apical Na^+/H^+ exchanger, rather than by the stimulation of electrolyte secretion. Our study shows that the effect of UGN on pH_i is GC-C/cGMP-mediated and involves the enzyme PDE5. In addition, this exposure results in intracellular alkalinization, probably due to indirect effects on basolateral NHE1 and NHE4 isoforms, which have a major role in pH_i regulation.

Acknowledgements

The authors thank Dr. Mariano Levin, Dr. Cristina Juarez and Lic. Laura Toledo for their helpful assistance. This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina), Universidad de Buenos Aires (UBA, Argentina) and the Agencia Nacional de Promoción Científica y Tecnológica (FONCyT, Argentina).

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