Escherichia coli Heat-Stable Enterotoxin Mediates Na⁺/H⁺ Exchanger 4 Inhibition Involving cAMP in T₈⁴ Human Intestinal Epithelial Cells

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Abstract

The enterotoxigenic Escherichia coli strains lead to diarrhoea in humans due to heat-labile and heat-stable (STa) enterotoxins. STa increases Cl-release in intestinal cells, including the human colonic carcinoma T₈⁴ cell line, involving increased cGMP and membrane alkalization due to reduced Na⁺/H⁺ exchangers (NHEs) activity. Since NHEs modulate intracellular pH (pHi), and NHE1, NHE2, and NHE4 are expressed in T₈⁴ cells, we characterized the STa role as modulator of these exchangers. pHi was assayed by the NH₄Cl pulse technique and measured by fluorescence microscopy in BCECF-preloaded cells. pHi recovery rate (dpHi/dt) was determined in the absence or presence of 0.25 μmol/L STa (30 minutes), 25 μmol/L HOE-694 (concentration inhibiting NHE1 and NHE2), 500 μmol/L sodium nitroprusside (SNP, spontaneous nitric oxide donor), 100 μmol/L dibutyryl cyclic GMP (db-cGMP), 100 nmol/L H89 (protein kinase A inhibitor), or 10 μmol/L forskolin (adenylyl cyclase activator). cGMP and cAMP were measured in cell extracts by radioimmunoassay, and buffering capacity (βi) and H⁺ efflux (JH⁺) was determined. NHE4 protein abundance was determined by western blotting. STa and HOE-694 caused comparable reduction in dpHi/dt and JH⁺ (~63%), without altering basal pHi (range 7.144–7.172). STa did not alter βi value in a range of 1.6 pH units. The dpHi/dt and JH⁺ was almost abolished (~94% inhibition) by STa + HOE-694. STa effect was unaltered by db-cGMP or SNP. However, STa and forskolin increased cAMP level. STa-decreased dpHi/dt and JH⁺ was mimicked by forskolin, and STa + HOE-694 effect was abolished by H89. Thus, incubation of T₈⁴ cells with STa results in reduced NHE4 activity...
leading to a lower capacity of pH recovery requiring cAMP, but not cGMP. STa effect results in a causal phenomenon (STa/increased cAMP/increased PKA activity/reduced NHE4 activity) ending with intracellular acidification that could have consequences in the gastrointestinal cells function promoting human diarrhoea.

**Introduction**

Intestinal colon cells are polarized epithelial cells that express a wide range of plasma membrane transporters for a variety of substrates. Membrane transporters at the apical border of these cells promote absorption and release of nutrients, electrolytes and water from and to the intestinal lumen. However, membrane transporters at the basolateral border maintain cell homeostasis by the release of these and other nutrients to the interstitium. The apical membrane of intestinal colon cells is directly exposed to agents and toxins, including the enterotoxigenic *Escherichia coli* (ETEC) strains, an intestinal agent leading to diarrhoea in humans [1]. ETEC colonizes host intestines and releases heat-labile and/or heat-stable (STa) enterotoxins. STa causes secretory diarrhoea and is responsible for about half of all ETEC–related diarrhoeal diseases, including traveller’s diarrhoea and epidemic diarrhoea of the newborn [1–5].

STa binds to guanylyl cyclase-C (GC-C) receptors expressed in intestine, kidney, testis and lung, leading to an increase in the intracellular cGMP level [6–8]. STa also increases chloride secretion in a cAMP–dependent manner via the cystic fibrosis transmembrane conductance regulator (CFTR) channels in rat jejunum [9]. In an early study, STa was shown to cause mucosal alkalization due to inhibition of the Na⁺/H⁺ exchange in rat duodenum [10,11]. However, there are not reports addressing whether this enterotoxin modulates intracellular pH (pHi), and whether this phenomenon would involve Na⁺/H⁺ exchangers (NHEs) activity. Since both cGMP and cAMP decrease NHEs activity [12,13], an increase in the intracellular pH (pHi) in response to STa is expected.

NHEs are key in the modulation of intracellular pH (pHi), and are differentially expressed and regulated in intestine epithelial cells [14–17]. At least 11 isoforms of the NHEs family have been identified, out of which NHE1, 2, 3, and 4 are expressed in gastrointestinal membranes [16,17]. NHE4 is highly expressed in the stomach, renal cortex and medulla, ureter, skeletal muscle, heart, liver, and spleen [18]. NHE4 is involved in gastric secretion [19] and plays a large role in controlling pH [20]. Indeed, NHE4 was identified in the human colon carcinoma cell line T₈₄ [21] and in human colonic crypts [13]. This exchanger isoform modulates plays a determinant role in maintaining pH homeostasis; however, nothing is known about the regulation of NHE4 activity in T₈₄ cells by ETEC–released STa. Since T₈₄ cells express the GC-C receptors for STa [22], we hypothesize that STa modulates NHE4 activity and the signalling pathways involved in this phenomenon in this cell type. Our findings suggest that STa decreases NHE4 activity, without altering its protein expression via a mechanism that requires cAMP. This could be determinant in the planning of future therapies for human diarrhoea.

**Materials and Methods**

**Cell culture**

The cell line T₈₄ derived from colonic adenocarcinoma of male adult human were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and used for the experiments. T₈₄ cells in culture (5% CO₂, 37°C, pH 7.4) were maintained in Dulbecco’s
modified Eagle’s medium F12 (DMEM/F12, Gibco, Grand Island, NY, USA) containing low (5 mmol/L) D-glucose and supplemented with 14.5 mmol/L NaHCO₃, 3.2 mmol/L D-glutamine, 15 mmol/L HEPES, 5% foetal calf serum (FCS), 100 IU/mL penicillin and 100 mg/mL streptomycin (hereafter referred as primary culture medium (PCM)) as described [21]. Cells were harvested with trypsin/EGTA (0.25/0.2%, 3 minutes, 37°C) and seeded on sterile glass coverslips or 24 well plates for further 72 hours culture until confluence. Cells were then rinsed (3 times) with PCM containing 0.2% FCS (low-FCS/PCM) and cultured in this medium for further 48 hours in order to obtain a cell cycle synchronized culture.

Measurement of pHi

T₈₄ cell monolayers in a glass coverslip were mounted in a thermoregulated chamber on an inverted microscope (Nikon Diaphot-TMD, Tokyoi, Japan). The cells were incubated for 10 minutes at 37°C with the fluorescent pH sensitive probe 2,7-bicarboxyethyl-5,6-carboxyfluorescein acetoxyethyl ester (BCECF-AM, 12 μmol/L) (Molecular Probes, Eugene, OR, USA), as described [21]. Cells were then superfused by gravity at 3 mL/minute (37°C) with the control solutions (CS) ((mmol/L) NaCl 141, KCl 5, CaCl₂ 1, KH₂PO₄ 0.4, MgCl₂ 0.5, MgSO₄ 0.4, Na₂HPO₄ 0.3, HEPES 10, D-glucose 0.6 (pH 7.4, 37°C)) using an electromechanic switching system (Heater and Valve Controller, Yale University Electronics Shop, New Haven, CT, USA). The pH was calculated from fluorescence ratios measured at excitation of 495/440 nm and emission at 520 nm using a Georgia Instruments PMT-400 photomultiplier system, as described [23]. An area of 260 μm diameter was read, including approximately 200–300 cells. Measurements were performed at 2.5–seconds interval for a period of 300 milliseconds per measurement. The pHi was calibrated using 10 μmol/L nigericin in a calibrating solution ((mmol/L) KCl 130, NaCl 20, CaCl₂ 1, MgCl₂ 1, HEPES 5 (pH 6.0, 7.0 and 8.0)) as described [21].

pHi recovery

The pHi recovery was examined by applying the NH₄Cl pulse technique [21,23,24]. In brief, BCECF-AM loaded cells were superfused with CS until the basal pHi was stabilized (~15 minutes). T₈₄ cells were preincubated with 0.1, 0.25 or 0.75 μmol/L STa for 30 minutes in the presence of 25 μmol/L HOE-694 (a concentration that inhibits NHE1 and NHE2 activity), as described [21,25,26]. The cells were then exposed (2 minutes) to CS supplemented with NH₄Cl (NH₄Cl/CS solution) ((mmol/L) NaCl 121, KCl 5.4, CaCl₂ 1, KH₂PO₄ 0.4, MgCl₂ 0.5, MgSO₄ 0.4, Na₂HPO₄ 0.3, HEPES 10, D-glucose 0.6, NH₄Cl 20 (pH 7.4, 37°C)). After this incubation period the NH₄Cl/CS solution was replaced by rinsing the cells with CS free of NH₄Cl, without or with 25 μmol/L HOE-694, 500 μmol/L sodium nitroprusside (SNP, spontaneous nitric oxide donor) [27], 100 μmol/L dibutyryl cyclic GMP (db-cGMP), 100 nmol/L H89 (a protein kinase A inhibitor) [28] or 10 μmol/L forskolin (an activator of adenyl cyclase) [29].

Initial rates of pHi recovery (dpHi/dt) were calculated from data collected for the first 60 seconds of the recovery (i.e., after removing the NH₄Cl load) and fitted by a first order lineal regression as described [21,24]. The results were expressed in pHi units/minute. The fraction of dpHi/dt mediated by NHE4 (dhpHi/dt) was estimated by the expression:

\[
\text{dhpHi/dt} = \left( \frac{\text{Total dpHi/dt}}{\text{HÖE dpHi/dt}} \right) - \left( \frac{\text{Total dpHi/dt}}{\text{HÖE dpHi/dt}} \right)
\]

where Total dpHi/dt is the dpHi/dt estimated in the absence of HOE-694 (i.e., total initial rate), and HÖE dpHi/dt is the dpHi/dt estimated in the presence of HOE-694, i.e., under inhibition of NHE1 and NHE2 [21]. The relative effect of STa on dhpHi/dt (STa RE) was determined by
the expression:

$$STa^{RE} = 100 \times \left( \frac{STaNHE_{dpHi/\Delta t}}{NHE_{dpHi/\Delta t}} \right)$$

where $STa^{NHE4}_{dpHi/\Delta t}$ is $NHE4_{dpHi/\Delta t}$ measured in the presence of STa.

Intrinsic buffering capacity

The ability of intrinsic cellular components to buffer changes in pH, i.e., intracellular buffer capacity ($B_i$), was measured as described [21,24]. After determining the basal pH, the cells were incubated in a 0.5 mmol/L KCl-containing Na⁺-free CS (Na⁺/CS) ((mmol/L) N-methyl-D-glucamine (NMDG) 120, KCl 5, CaCl₂ 1.8, MgCl₂ 1, HEPES 30, D-glucose 5 (pH 7.4, 37°C)). Cells were then incubated in the latter solution containing decreasing concentrations of NH₄Cl (50, 20, 10, 5, 2.5 or 1 mmol/L). The $B_i$ ($Beta(i)$) was calculated from the expression:

$$Beta(i) = \frac{\text{change} \left[ NH_4^+ \right]}{\text{change} \left( \text{pHi} \right)}$$

where the intracellular NH₄⁺ concentration ([NH₄⁺]i) was obtained from the Henderson-Hasselbalch equation on the assumption that [NH₃]i (intracellular NH₃) was equivalent to [NH₃]o (extracellular NH₃), and change (pHi) is the fraction of change in units of pH value. Knowing the $dpHi/\Delta t$ and $B_i$ values, the rate of overall transmembrane H⁺ flux ($J_{H^+}$) was calculated from the following expression:

$$J_{H^+} = Beta(i) \times \left( \frac{dpHi}{dt} \right)$$

cAMP and cGMP determination

T₈₄ cells were cultured to confluence in 96-well plates. Cells were first treated for 10 minutes with 1 mmol/L 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, St. Louis, MO, USA) and next incubated for another 10 minutes with culture medium containing IBMX or IBMX and STa or forskolin. cAMP and cGMP levels were measured by enzyme immunoassay (cAMP or cGMP Direct Biotrak EIA, GE Healthcare, PA, USA) according to manufacturer's instructions. Values of cAMP or cGMP were normalized to total cell protein per well.

Western blotting

Total protein was obtained from confluent T₈₄ cells rinsed (x2) with ice-cold PBS and harvested in 100 μL of lysis buffer (10% SDS, 20% glycerol, 100 mmol/L dithiothreitol, 2.9 mmol/L Tris (pH 6.8), 0.1% bromophenol blue) (63.7 mmol/L Tris/HCl (pH 6.8), 10% glycerol, 2% sodium dodecylsulphate, 1 mmol/L Na₃VO₄, 50 mg/mL leupeptin, 5% β-mercaptoethanol) as described [21,27]. Cells were sonicated (6 cycles, 5 seconds, 100 W, 4°C) and total protein was isolated by centrifugation (13500 g, 15 minutes, 4°C). Proteins (50 μg) were separated by polyacrylamide gel (7.5%) electrophoresis, transferred to Immobilon-P polyvinylidene difluoride membranes (BioRad Laboratories, Hertfordshire, UK) and probed with primary monoclonal rabbit anti-NHE1 (1:1000 dilution, 12 hours, 4°C), primary polyclonal rabbit anti-NHE2 (1:1000 dilution, 12 hours, 4°C) (Abcam, Cambridge, UK), primary rat anti-NHE4 antibody (11H11, amino acids 565–675, ~55 kDa) (kindly donated by Dr Daniel Biemesderfer from Yale School of Medicine, New Haven, CT, USA) (1:1000 dilution, 2 hours, 22°C), or monoclonal mouse anti-β-actin (1:5000 dilution, internal reference) (Santa Cruz Biotechnology, Santa
Cruz, CA, USA) antibodies. The membranes were rinsed in Tris buffer saline-0.1% Tween 20 (TBS-T) and further incubated (1 hour) in TBS-T/0.2% bovine serum albumin (BSA) containing secondary horseradish peroxidase-conjugated goat anti-rat or anti-mouse antibodies (Thermo Scientific, Rockford, IL, USA). Proteins were detected by enhanced chemiluminescence (film exposure time was 1 minute) in a ChemiDoc-It 510 Imagen System (UVP, LCC Upland, CA, USA) and quantified by densitometry [27,30].

Statistical analysis
The values are mean ± S.E.M., where n indicates number of different cell cultures (n = 27 for STa-untreated (i.e., control) and 25 STa-treated cells) with 3–4 replicates per experiment. The normality of the data (i.e., parametric) was confirmed with Kolmogorov-Smirnov’s test. The variances across the control and STa-treated cells under Bartlett’s test were homogeneous. Comparisons between two groups were performed by means of Student’s unpaired t-test. The difference between more than two groups were performed by analysis of variance (ANOVA, one or two-ways). If the ANOVA demonstrated a significant interaction between variables, post hoc analyses were performed by the multiple-comparison Bonferroni test. The experimenter running the assays was blinded to the groups allocation before and during the experiments, and when assessing the outcome (i.e., around 30 days). The statistical software GraphPad InStat 3.0b and GraphPad Prism 7.0a.65 (GraphPad Software Inc., San Diego, CA, USA) were used for data analysis. P<0.05 was considered statistically significant.

Results
Effect of STa on pH\textsubscript{i} values
Basal pH\textsubscript{i} in T\textsubscript{84} cells detected in this study was comparable to previous reports in this cell type [21,31,32] and was unaltered in cells preincubated with STa (Table 1, Fig 1). Following the NH\textsubscript{4}Cl pulse the acidic pH\textsubscript{i} values detected in the cells exposed to STa or HOE-694 were partially restored (27 ± 3 or 55 ± 6%, respectively) compared with cells in the absence of these agents (Fig 1). When cells were coincubated with STa + HOE-694 the NH\textsubscript{4}Cl-induced acidic pH\textsubscript{i} was only minimally restored (9 ± 1%).

Effect of STa on pH\textsubscript{i} recovery kinetics
Since T\textsubscript{84} cells express NHE1, NHE2 and NHE4, but not NHE3 forms [21,33], we assayed which of these forms was involved in STa effect on \(dpH\textsubscript{i}/dt\). The \(dpH\textsubscript{i}/dt\) values in the presence of STa or HOE-694 were lower (65 ± 7 or 62 ± 6%, respectively) when compared with cells in the absence of these molecules (Table 1). Coincubation of cells with STa + HOE-694 resulted in higher reduction (90 ± 6%) in the \(dpH\textsubscript{i}/dt\) compared with the reduction seen in cells treated with STa or HOE-694 alone.

Effect of STa on \(\beta\textsubscript{i}\) and \(J\textsubscript{H\textsuperscript{+}}\)
The \(\beta\textsubscript{i}\) value detected in T\textsubscript{84} cells in the absence of STa (31.1 ± 2.5 (mmol/L)/intracellular pH units) was similar to that previously reported for this cell type under the same culture and measurement conditions (~31 (mmol/L)/intracellular pH units) [21]. Change in \(\beta\textsubscript{i}\) value was not significantly altered by 0.25 μmol/L STa in a range of 1.6 pH\textsubscript{i} units in T\textsubscript{84} cells. Parallel assays show that cells treated with STa exhibit decreased \(J\textsubscript{H\textsuperscript{+}}\) (60 ± 7%) compared with cells in the absence of this toxin (Fig 2A). Since maximal inhibitory effect on this parameter was achieved with 0.25 μmol/L STa in the presence of 25 μmol/L HOE-694 (Fig 2B), this concentration was used in all subsequent experiments. HOE-694 caused a decrease in \(J\textsubscript{H\textsuperscript{+}}\) (56 ± 7%) that was
similar (P > 0.05) to that in cells in the presence of STa. Coincubation of cells with STa + HOE-694 resulted in a decrease in J_{1H}^+ (89 ± 6%) that was higher compared with the effect seen in cells treated with STa or HOE-694 alone.

NHE1, NHE2 and NHE4 protein abundance

To address whether STa–associated decrease in J_{1H}^+ was due to lower protein abundance of NHE4, or whether this toxin alters NHE1 or NHE2 protein abundance, the protein level of these membrane transporters was assayed. The results show that incubation of T_{84} cells with STa did not alter NHE1, NHE2 or NHE4 protein abundance (Fig 3).

cGMP and cAMP involvement on NHE4–mediated pH_{i} recovery kinetics

STa is shown to increase the cGMP level in T_{84} cells [34]; however, the role of cGMP as modulator of NHE4 activity is not addressed [17]. Thus, we next investigated whether STa effect on
NHE4–dependent \( \text{dpHi}/dt \) in this cell type was modulated by direct administration of exogenous cGMP. The results show that \( \text{dpHi}/dt \) and basal pHi (Table 1), and \( J_{\text{H}^+} \) (Fig 4) were unaltered in T84 cells exposed to db-cGMP in the absence of HOE-694 or STa. However, the reduction in \( \text{dpHi}/dt \) and \( J_{\text{H}^+} \) seen in response to STa, HOE-694, or STa + HOE-694 was unaltered by db-cGMP. When cells were incubated with SNP (a spontaneous NO donor) [27] the results were similar to those in the presence of db-cGMP (Table 1, Fig 4). Parallel results show that cGMP intracellular level was increased by STa and SNP, confirming previous reports in T84 cells [35] and rat distal colon crypts [36], but it was unaltered by HOE-694 (not shown).

We next assayed whether CAMP was involved in the response of T84 cells to STa–reduced NHE4–mediated pH recovery kinetics. Cells incubated with forskolin (adenylyl cyclase activator) [29] in the absence of HOE-694 resulted in a decrease in \( \text{dpHi}/dt \) (Table 1) and \( J_{\text{H}^+} \) (Fig 5A) that was of a similar magnitude to the decrease seen in cells incubated with STa in the absence or presence of this activator. However, in the presence of HOE-694 or STa + HOE-694, forskolin caused a reduction in these parameters that was similar to that seen in cells coincubated with STa + HOE-694 in the absence of this activator. Parallel results show that intracellular level of cAMP increased by STa (4.9 ± 0.5 fold) and forskolin (8.9 ± 1.5 fold) (Fig 5B). Additionally, pre-incubation of cells with H89 (inhibitor of PKA) [28] reversed the decrease in \( \text{dpHi}/dt \) and \( J_{\text{H}^+} \) caused by STa + HOE-694 to values that are comparable to STa or HOE-694 alone.

**Discussion**

This study shows that the enterotoxigenic *Escherichia coli* (ETEC) released heat-stable (STa) enterotoxin decreases the pH recovery kinetics in the human colonic carcinoma T84 cell line. This
phenomenon results from a lower activity of NHE4 without altering its protein expression. STa effect depends on the level of cAMP, but not cGMP, and PKA activation. These findings represent a novel mechanism of pHi homeostasis by STa that could have consequences in the physiology of gastrointestinal cells leading to human diarrhoea.

**STa modulation of NHEs activity**

STa is an enterotoxin that causes gastrointestinal electrolyte imbalance characterized by a higher Cl− release to the gastrointestinal lumen, a phenomenon that ends in diarrhoea in humans [1,3–5]. One of the potential mechanisms for these adverse effects of STa is a mucosal alkalization due to lower activity of plasma membrane mechanisms involved in maintaining transmembrane distribution of H+ including NHEs activity [10,11]. Our results show that STa caused a decrease
in NHEs activity resulting in lower H⁺ efflux (i.e., \( J_{\text{H}^+} \)). This phenomenon may be responsible for the observed reduction in the capacity to restore the pHi recovery kinetics after an acid pulse. This possibility is supported by the findings showing that STa caused a similar reduction in \( dp\text{Hi}/dt \) and \( J_{\text{H}^+} \) (reduction in \( dp\text{Hi}/dt \) / reduction in \( J_{\text{H}^+} \) = 1.1), thus, making possible that alterations in the pHi recovery rate caused by STa was due to reduced H⁺ efflux kinetics. In addition, since the intrinsic buffering capacity (\( \beta_i \)) values were unaltered by STa (\( \beta_i \) with STa/\( \beta_i \) without STa = 1), it is unlikely that these alterations were the result of an altered \( \beta_i \) in T84 cells. Indeed, in cells incubated with STa the pHi value was not significantly altered (pHi with STa/pHi without STa = 0.996) compared with cells in the absence of this toxin.

Fig 3. Effect of STa on NHE4 protein abundance. Western blot for NHE4 protein abundance in whole extracts of T84 cells exposed for 30 minutes in the absence (Control) or presence (STa) of 0.25 μmol/L heat-stable (STa) enterotoxin. Lower panel: NHE4/β-actin ratio densitometries normalized to 1 in Control. β-Actin is internal reference. Values are mean ± S.E.M. (n = 15).

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Fig 4. Involvement of cGMP on STa modulation of \( J_{\text{H}^+} \). T84 cells were exposed for 30 minutes in the absence (– SNP) or presence (+ SNP) of 500 μmol/L sodium nitroprusside (SNP). The overall transmembrane H⁺ flux rates (\( J_{\text{H}^+} \)) were calculated from initial rates of pHi recovery and the intrinsic buffer capacity (\( \beta_i \)) values (see Methods). Cells were exposed to culture medium without (–, Control, red bar) or with (+) 100 μmol/L dibutyryl cyclic GMP (db-cGMP), 0.25 μmol/L STa, and/or 25 μmol/L HOE-694 (see Methods). *\( P<0.05 \) versus Control or corresponding db-cGMP, †\( P<0.05 \) versus corresponding STa or HOE-694 in the presence of db-cGMP. Values are mean ± S.E.M. (n = 25–27).

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Interestingly, it was initially shown [31] that T84 cells express mainly NHEs (NHE1, NHE2 and NHE4) [21], in a minor grade Cl⁻/HCO₃⁻ exchangers and Na⁺/HCO₃⁻ cotransporters, but not other classical mechanisms of H⁺ export such as the vacuolar H⁺-ATPases [37] or H⁺/K⁺-ATPases [38]. Out of these membrane transport systems, NHEs play a major role in the removal of intracellular H⁺ in most cell types maintaining stable pHᵢ and extracellular pH values [14–17,20,37,38].

NHE4 is an isoform of the NHEs family of membrane exchangers whose function results in the modulation of pHᵢ in mammalian cells [14,16,17]. This membrane Na⁺/H⁺ exchanger isoform is expressed in the human gastrointestinal tract, and is co-expressed with NHE1 and...
NHE2, but not NHE3, in T84 cells [21,33], as confirmed in this study. Interestingly, cells exposed to HOE-694 show lower \( \frac{dpH}{dt} \) and \( J_{\text{H}^+} \) most likely via a mechanism involving lower activity of NHE1 and NHE2 isoforms, since the concentration of this inhibitor used in the present study (25 \( \mu \)mol/L) preferentially inhibits these isoforms, but not NHE4 [21,26]. Indeed, cells in the presence of HOE-694 show partial recovery of the pH\(_i\) value suggesting that not all the pH\(_i\) recovery is mediated by NHE1 and NHE2, but other mechanism(s) is plausible in this cell type.

Since STa in the presence of HOE-694, i.e., where NHE1 and NHE2 were not functional, almost abolished the \( \frac{dpH}{dt} \) and \( J_{\text{H}^+} \) (both reduced by \( \sim 90\% \)), it is likely that NHE4 isoform was inhibited by this enterotoxin in T84 cells. This possibility is supported when we consider that the concentration of STa used in our study is close to the STa half-maximal stimulatory concentration for cGMP accumulation reported in T84 cells [25]. Additionally, the possibility that STa reduces the \( \frac{dpH}{dt} \) and \( J_{\text{H}^+} \) via a mechanism including lower expression of NHE4, or NHE1 or NHE2, is unlikely since the protein abundance for none of these isoforms were altered by the toxin. Thus, STa–reduced H\(^+\) efflux seems to be due to a lower activity rather than expression of NHE4 in this cell type. STa effect in the presence of HOE-694 leads a remaining fraction of pH\(_i\) recovery that accounted for 10% of the total recovery after an acid pulse. This finding could results from other mechanisms than inhibition of NHE1, 2 or 4, such as activity of Cl\(^-\)/HCO\(_3^-\) exchangers and/or Na\(^+\)/HCO\(_3^-\) cotransporters expressed in T84 cells [31]. Indeed, STa was shown to increase HCO\(_3^-\) secretion via a higher Na\(^+\)/HCO\(_3^-\) activity in duodenal CFTR\(^{+/−}\) mice [39]. However, our pH\(_i\) recovery assays were performed in the absence of extracellular HCO\(_3^-\) making the latter unlikely.

**Involvement of cAMP on STa effect**

It has been shown that STa increases Cl secretion in a cAMP–and cGMP–dependent manner via CFTR channels in rat jejunum [9]. Initial reports show that STa–increased cGMP, but unaunted cAMP level in rabbit distal ileum mucosa [40] or reduced cAMP level in mice intestine [41]. Our results show that exposure of T84 cells to STa results in increased cGMP and cAMP levels. Since these nucleotides decrease NHEs activity [12,13], STa–increased levels may have functional consequences on pH\(_i\) recovery in T84 cells.

Since incubation of cells with exogenous cGMP (db-cGMP) did not alter basal \( \frac{dpH}{dt} \) and \( J_{\text{H}^+} \) in our assays it is likely that this cyclic nucleotide is not involved in the modulation of NHEs activity in T84 cells. Furthermore, the inhibitory effect of STa on \( \frac{dpH}{dt} \) and \( J_{\text{H}^+} \) in the presence of HOE-694 was unaltered by db-cGMP, suggesting that NHE4 inhibition by STa was independent of cGMP. This is supported by the findings showing that \( \frac{dpH}{dt} \) and \( J_{\text{H}^+} \) inhibition by STa or HOE-694 alone was unaltered when cells were coincubated with these molecules and db-cGMP. Additionally, exposure of cells to exogenous NO delivered by SNP, a spontaneous NO donor [27], does not change STa effect in the absence or presence of HOE-694. Since SNP did not alter the reduction in the \( \frac{dpH}{dt} \) and \( J_{\text{H}^+} \) caused by HOE-694 itself, NO in this cell type may not alter this inhibitors’ effectiveness on NHE1 and NHE2.

It was early shown that forskolin, a potent activator of adenylyl cyclase, has a profound effect in T84 transmonolayer net water flux (\( J_w \)) [29], suggesting that cAMP could be involved in this phenomenon. Unfortunately, the cAMP level was not determined in the latter study. Additionally, incubation of T84 cells with secretagogues whose actions are mediated by cAMP ends with Cl secretion from this cell type [35,42–44]. However, it is paradoxical that even when the level of cAMP was found unaltered in T84 cells in response to STa, this toxin effect on Cl secretion closely resembles a cAMP–mediated mechanism in this cell type [35]. Our findings show that cAMP level is increased in T84 cells treated with STa or with forskolin. Since the
The effect of forskolin alone was to diminish the $\frac{dpHi}{dt}$ and $J^{+}_{H}$ in a same magnitude as STa alone or STa + forskolin, it is likely that a higher cAMP level could be involved in downregulation of NHE4 activity in this cell type. Parallel results suggest that NHE1 and NHE2 may not be under modulation by STa–or forskolin–mediated cAMP increase since the inhibition caused by HOE-694 of $\frac{dpHi}{dt}$ and $J^{+}_{H}$ by itself or in the presence of STa was unaltered by forskolin. Interestingly, since H89, a PKA inhibitor, resulted in restoration of the reduced $\frac{dpHi}{dt}$ and $J^{+}_{H}$ seen in the presence of STa + HOE-694 + forskolin to values that are comparable to those in the presence of these molecules per separate, it is likely that PKA may mediate STa inhibition of NHE4 in T84 cells.

In conclusion, the enterotoxigenic Escherichia coli released heat-stable enterotoxin (STa) has a deleterious effect on the normal physiology of T84 cells in vitro. In terms of its association with human diarrhoea this enterotoxin was found to increase not only cGMP levels, but also the cAMP level, perhaps leading to PKA activation in this cell type. It is proposed that STa reduces the capacity of T84 cells to recover the pH, after an acid pulse via a mechanism that includes reduced activity of NHE4, but not NHE1 or NHE2, in this cell type. These findings constitute a novel mechanism of pH homeostasis by STa in this cell type, and perhaps in the...
gastrointestinal epithelium, resulting in a deficient recovery rate and H+ efflux after metabolic alterations associated with intracellular acidification. These findings complement the reduced transepithelial electrical resistance caused by STa in T84 cells, indicative of an intestinal barrier dysfunction in addition to STa–induced water secretion [45]. Considering that T84 cells respond with increased CI release to STa via cGMP–and cAMP–dependent mechanisms, a role of NHE4 is this phenomenon is proposed. All together the alterations caused by STa in a functional sequence (i.e., STa / increased cAMP / increased PKA activity / decreased NHE4 activity / increased intracellular acidification) (Fig 6) could have consequences in the physiology of gastrointestinal cells promoting human diarrhoea.

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Author Contributions
Conceived and designed the experiments: ARB GM LS MAR. Performed the experiments: ARB LRC-L CNAB MC JA FP AL KN. Analyzed the data: ARB FT JA FP AL CS GM LS MAR KN. Contributed reagents/materials/analysis tools: CS FP AL FT GM LS MAR. Wrote the paper: ARB MC LS MAR.

References
1. Qadri F, Svennerholm AM, Faruque AS, Sack RB. Enterotoxigenic Escherichia coli in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin Microbiol Rev. 2005; 18: 465–483. PMID: 16020685
2. Gross R. Escherichia coli diarrhoea. J Infect. 1983; 7: 177–192. PMID: 6363562
3. Cravioto A, Reyes R, Ortega R, Fernandez G, Hernandez R, Lopez D. Prospective study of diarrhoeal disease in a cohort of rural Mexican children: incidence and isolated pathogens during the first two years of life. Epidemiol Infect. 1988; 101: 123–134. PMID: 3402544
4. Abu-Elyazeed R, Wierzbza A, Mourad L, Peruski B, Kay BA, Rao M, et al. Epidemiology of enterotoxigenic Escherichia coli diarrhoea in a pediatric cohort in a periurban area of lower Egypt. J Infect Dis. 1999; 179: 382–389. PMID: 9878022
5. Moon C, Zhang W, Sundaram N, Yarlagadda S, Reddy VS, Arora K, et al. Drug-induced secretory diarrhoea: A role for CFTR. Pharmacol Res. 2015; 102: 107–112. doi: 10.1016/j.phrs.2015.08.024 PMID: 26429773
6. Forte LR, Krause WJ, Freeman RH. Escherichia coli enterotoxin receptors: localization in opossum kidney, intestine, and testis. Am J Physiol. 1989; 257: F874–F881. PMID: 2556042
7. Schulz S, Green CK, Yuen PS, Garbers DL. Guanylyl cyclase is a heat-stable enterotoxin receptor. Cell 1990; 63: 941–948. PMID: 1701694
8. Carrithers SL. Diarrhea or colorectal cancer: can bacterial toxins serve as a treatment for colon cancer?. Proc Natl Acad Sci USA. 2003; 100: 3018–3020. PMID: 12631696
9. Golin-Bisello F, Bradbury N, Ameen N. STa and cGMP stimulate CFTR translocation to the surface of villus enterocytes in rat jejunum and is regulated by protein kinase G. Am J Physiol. 2005; 289: C708–C716.
10. Fawcus K, Gorton VJ, Lucas ML, McEwan GT. Stimulation of three distinct guanylate cyclases induces mucosal surface alkalisation in rat small intestine in vitro. Comp Biochem Physiol A Physiol. 1997; 118: 291–295. PMID: 936058
11. Lucas ML. A reconsideration of the evidence for Escherichia coli STa (heat stable) enterotoxin-driven fluid secretion: a new view of STa action and a new paradigm for fluid absorption. J Applied Microbiol. 2001; 90: 7–26.
12. Bachmann O, Juric M, Seidler U, Manns M, Yu H. Basolateral ion transporters involved in colonic epithelial electrolyte absorption, anion secretion and cellular homeostasis. Acta Physiol. 2011; 201: 33–46.
13. Arena E, Longo W, Roberts K, Geibel P, Nateqi J, Brandstetter M, et al. Functional role of NHE4 as a pH regulator in rat and human colonic crypts. Am J Physiol. 2012; 302: C412–C418.
14. Slepkov ER, Rainey JK, Sykes BD, Fliegel L. Structural and functional analysis of the Na+/H+ exchanger. Biochem J. 2007; 401: 623–633. PMID: 17209804
15. Orlowski J, Grinstein S. Na+/H+ exchangers. Compr Physiol. 2011; 1: 2083–2100. doi:10.1002/cphy.c110020 PMID: 23736988
16. Provost JJ, Wallert MA. Inside out: targeting NHE1 as an intracellular and extracellular regulator of cancer progression. Chem Biol Drug Des. 2013; 81: 85–101. doi:10.1111/cbdd.12035 PMID: 23253131
17. Fuster DG, Alexander RT. Traditional and emerging roles for the SLC9 Na+/H+ exchangers. Pflugers Arch. 2014; 466: 61–76. doi:10.1007/s00424-013-1408-8 PMID: 24337822
18. Ikuma M, Kashgarian M, Binder HJ, Rajendran VM. Differential regulation of NHE isoforms by sodium depletion in proximal and distal segments of rat colon. Am J Physiol. 1999; 276: G539–G549. PMID: 9950829
20. Rossmann H, Sonnentag T, Heinzmann A, Seidler B, Bachmann O, Vieillard-Baron D, et al. Differential expression and regulation of Na+/H+ exchanger isoforms in rabbit parietal and mucous cells. Am J Physiol. 2001; 281: G447–G458.
21. Beltran AR, Ramirez MA, Carraro-Lacroix LR, Hiraki Y, Reboucas NA, Malnic G. NHE1, NHE2, and NHE4 contribute to regulation of cell pH in T84 colon cancer cells. Pflugers Arch. 2008; 455: 799–810. PMID: 17943310
23. Fernandez R, Malnic G. H+ ATPase and Cl interaction in regulation of MDCK cell. J Membr Biol. 1998; 163: 137–145. PMID: 992078
24. Aravena C, Beltran AR, Cornejo M, Torres V, Diaz ES, Guzmán-Gutiérrez, et al. Potential role of sodium-proton exchangers in the low concentration arsenic trioxide-increased intracellular pH and cell proliferation. PloS One 2012; 7: e51451. doi: 10.1371/journal.pone.0051451 PMID: 23236503
25. Ikuma M, Kashgarian M, Binder HJ, Rajendran VM. Differential regulation of NHE isoforms by sodium depletion in proximal and distal segments of rat colon. Am J Physiol. 1999; 276: G539–G549. PMID: 9950829
27. Pardo F, Silva L, Sáez T, Salsoso R, Guzmán-Gutiérrez J, Sanhueza C, et al. Human supraphysiological gestational weight gain and fetoplacental vascular dysfunction. Int J Obes (Lond). 2015; 39: 1264–1273.
28. Ao M, Sarathy J, Domingue J, Alrefai WA, Rao MC. Chenodeoxycholic acid stimulates Cl- secretion via cAMP signaling and increases cystic fibrosis transmembrane conductance regulator phosphorylation in T84 cells. Am J Physiol. 2013; 305: C447–C456.
29. Salsoso R, Guzmán-Gutiérrez E, Sáez T, Bugueno K, Ramirez MA, Farias M, et al. Insulin restores L-arginine transport requiring adenosine receptors activation in umbilical vein endothelium from late-onset preeclampsia. Placenta 2015; 36: 287–296. doi:10.1016/j.placenta.2014.12.007 PMID: 25573092
30. Ramírez MA, Toriano R, Parisi M, Malnic G. Control of cell pH in the T84 colon cell line. J Membr Biol. 2000; 177: 149–157. PMID: 11033689
31. Ramírez MA, Toriano R, Parisi M, Malnic G. Control of cell pH in the T84 colon cell line. J Membr Biol. 2000; 177: 149–157. PMID: 11033689
32. Musa-Aziz R, Oliveira-Souza M, Mello-Aires M. Signaling pathways in the biphasic effect of ANG II on Na+/H+ exchanger in T84 cells. J Membr Biol. 2005; 205: 49–60. PMID: 16263585
33. Toriano R, Ozu M, Polit1 MT, Dorr RA, Curto MA, Capurro C. Uroguanylin regulates net fluid secretion via the NHE2 isoform of the Na+/H+ exchanger in an intestinal cellular model. Cell Physiol Biochem. 2011; 28: 733–742. doi: 10.1159/000335767 PMID: 22178885
34. Kots AY, Choi BK, Estrella-Jimenez ME, Warren CA, Gilberston SR, Guerrant RL, et al. Pyridopyrimidine derivatives as inhibitors of cyclic nucleotide synthesis: Application for treatment of diarrhea. Proc Natl Acad Sci USA. 2008; 105: 8440–8445. doi: 10.1073/pnas.0803096105 PMID: 18559851
35. Huott PA, Liu W, McRoberts JA, Giannella RA, Dharmsathaphorn K. Mechanism of action of Escherichia coli heat stable enterotoxin in a human colonic cell line. J Clin Invest. 1988; 82: 514–523. PMID: 2457034

36. Morel E, Dublineau I, Griffiths NM. Effect of radiation on cAMP, cGMP and Ca2+i pathways and their interactions in rat distal colon. Radiat Res. 2003; 160: 263–272. PMID: 12926985

37. Casey JR, Grinstein S, Orlowski J. Sensors and regulators of intracellular pH. Nat Rev Mol Cell Biol. 2010; 11: 50–61. doi: 10.1038/nrm2820 PMID: 19997129

38. Gillies RJ, Raghunand N, Garcia-Martin ML, Gatenby RA. pH imaging. A review of pH measurement methods and applications in cancers. IEEE Eng Med Biol Mag. 2004; 23: 57–64.

39. Sellers ZM, Childs D, Chow JYC, Smith AJ, Hogan DL, Isenberg JI, et al. Heat-stable enterotoxin of Escherichia coli stimulate a non-CFTR-mediated duodenal bicarbonate secretory pathway. Am J Physiol. 2005; 288: G654–G663.

40. Field M, Graf LH, Laird WJ, Smith PL. Heat-stable enterotoxin of Escherichia coli: in vitro effects on guanylate cyclase activity, cyclic GMP concentration, and ion transport in small intestine. Proc Natl Acad Sci USA. 1978; 75: 2800–2804. PMID: 26915

41. Giannella RA, Drake KW. Effect of purified Escherichia coli heat-stable enterotoxin on intestinal cyclic nucleotide metabolism and fluid secretion. Infect Immun. 1979; 24: 19–23. PMID: 378842

42. Cartwright CA, McRoberts JA, Mandel KG, Dharmsathaphorn K. Synergistic action of cyclic adenosine monophosphate-and calcium-mediated chloride secretion in a colonic epithelial cell line. J Clin Invest. 1985; 76: 1837–1842. PMID: 2997291

43. Wasserman SI, Barrett KE, Huott PA, Beuerlein G, Kagnoff MF, et al. Immune-related intestinal Cl-secretion. I. Effect of histamine on the T84 cell line. Am J Physiol. 1988; 254: C53–C62. PMID: 3337221

44. Nichols JM, Maiellaro I, Abi-Jaoude J, Curci S, Hofer AM. “Store-operated” cAMP signaling contributes to Ca2+-activated Cl- secretion in T84 colonic cells. Am J Physiol. 2015; 309: G670–G679.

45. Nakashima R, Kamata Y, Nishikawa Y. Effects of Escherichia coli heat-stable enterotoxin and guanylin on the barrier integrity of intestinal epithelial T84 cells. Vet Immunol Immunopathol. 2013; 152: 78–81. doi: 10.1016/j.vetimm.2012.09.026 PMID: 23078906