Penicillin G Induces H$^+$, K$^+$-ATPase via a Nitric Oxide-Dependent Mechanism in the Rat Colonic Crypt

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Abstract

Background/Aims: The colonic H$^+$, K$^+$ ATPase (HKA2) is a heterodimeric membrane protein that exchanges luminal K$^+$ for intracellular H$^+$ and is involved in maintaining potassium homeostasis. Under homeostatic conditions, the colonic HKA2 remains inactive, since most of the potassium is absorbed by the small intestine. In diarrheal states, potassium is secreted and compensatory potassium absorption becomes necessary. This study proposes a novel mechanism whereby the addition of penicillin G sodium salt (penG) to colonic crypts stimulates potassium uptake in the presence of intracellular nitric oxide (NO), under sodium-free (0-Na$^+$) conditions.

Methods: Sprague Dawley rat colonic crypts were isolated and pHi changes were monitored through the ammonium prepulse technique. Increased proton extrusion in 0-Na$^+$ conditions reflected heightened H$^+$, K$^+$ ATPase activity. Colonic crypts were exposed to penG, L-arginine (a NO precursor), and N-nitro l-arginine methyl ester (L-NAME, a NO synthase inhibitor).

Results: Isolated administration of penG significantly increased H$^+$, K$^+$ ATPase activity from baseline, p 0.0067. Co-administration of arginine and penG in 0-Na$^+$ conditions further upregulated H$^+$, K$^+$ ATPase activity, p <0.0001. Crypt perfusion with L-NAME and penG demonstrated a significant reduction in H$^+$, K$^+$ ATPase activity, p 0.0058.

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Conclusion: Overall, acute exposure of colonic crypts to penG activates the H$^+$, K$^+$ ATPase in the presence of NO. This study provides new insights into colonic potassium homeostasis.

Keywords

(3–5) Fluid secretion; H$^+$, K$^+$ ATPase; Nitric oxide pathway; Penicillin G; Colon

Introduction

The H$^+$, K$^+$ ATPase (HKA) is an alpha, beta heterodimeric membrane protein that is subdivided into the gastric HKA1 and the non-gastric, HKA2 [1]. A member of the P-type ATPase family, H$^+$, K$^+$ ATPase exchanges luminal K$^+$ for intracellular H$^+$ ions against a concentration gradient [2]. While the function of gastric HKA1 has been well-characterized, the enzymatic properties of HKA2 are inconclusive [3–5].

The colonic HKA2 isoform has variable properties and sensitivities

The HKA2 is encoded by the ATP12A gene and has been detected in colonic tissues across mice, rats, rabbits, and humans [6–10]. As opposed to HKA1, HKA2 has a pharmacological profile that varies depending on the species and environment. Early studies evaluated whether HKA2 was sensitive to omeprazole (a proton pump inhibitor), SCH28080 (a potassium-competitive acid blocker), and ouabain (a Na$^+$, K$^+$ ATPase inhibitor) [10–19]. The results of these studies depended on whether the HKA2 was rat- versus human-derived or expressed in other cell lines. For example, HKA2 in the rat distal colon is insensitive to omeprazole yet partly inhibited by ouabain [11, 12]. Human HKA2 expressed in human embryonic kidney cells and Xenopus oocytes are both SCH28080- and ouabain-sensitive [13–15, 17].

The HKA2 not only has various sensitivity profiles, but also varies with regards to location. Along the rat and human colon, the HKA2 is distributed unevenly with the highest density in the distal colonic segments [10]. While ouabain-sensitive HKA2 cells are in the rat colonic crypts, ouabain-insensitive HKA2 activity exist in surface cells [12, 14, 16, 18–20].

HKA2's role in potassium absorption under fluid-constricted, low-potassium conditions

The role of HKA2 in potassium homeostasis has been evaluated in multiple studies. The importance of potassium regulation by HKA2 is unveiled in low-potassium conditions where the transporter is absent. HKA2 knockout mice placed on a Na$^+$ or K$^+$ free diet developed profound hypokalemia [21]. In contrast, wild-type mice placed on Na$^+$ or K$^+$ free diets do not experience hypokalemia [22]. Rats with high aldosterone levels had elevated HKA2 activity and potassium absorption. When fed low Na$^+$ diets, rats had increased aldosterone levels and elevated HKA2 mRNA and protein in apical membrane colonocytes [23, 24]. Mice injected with aldosterone over several days had active K$^+$ colonic absorption, suggesting that the gene coding for H$^+$, K$^+$ ATPase may be a target for aldosterone [25]. Of note, aldosterone activates the Na$^+$, K$^+$ ATPase, excreting potassium in exchange for sodium [26]. It is possible that HKA2 activation in fluid-constricted states is a type of rescue mechanism to prevent intestinal potassium loss that occurs through the Na$^+$, K$^+$ ATPase.

As a whole, net potassium absorption occurs in the small intestine, with only 9–10 milliequivalents of potassium being delivered to the distal colon per day [27, 28]. However, in pathological cases of fluid loss, colonic handling of potassium can become clinically significant depending on the type of diarrhea [28]. Diarrhea caused by ingestion of polyethylene glycol – an osmotically active agent unaltered by gut flora – leads to only small increases in fecal potassium losses [29]. However, other diarrheal types like secretory diarrhea lead to fecal potassium wasting in a diarrheal weight-dependent fashion [29]. Studies using Ussing chambers may be limited in evaluating potassium fluxes, since they are confined to the surface epithelium and may flush the potassium away before the transporter can absorb it [27]. With studies on colonic crypts, it is possible to isolate the most densely populated region of the HKA2 and to quantify the activity in isolation under sodium-free conditions.

PenG and NO's role in colonocyte fluid modulation

Antibiotic-associated diarrhea is thought to occur when alterations to the host microbiome lead to fluid and electrolyte losses. Previously, we perfused explanted rat intestines with a cocktail of antibiotics and found that there was increased fluid secretion over time [30]. Here, we evaluated whether antibiotics had a microbial independent effect on fluid and electrolyte status at the level of the host transporters. We measured transporter activity using the previously established ammonium prepulse technique [31, 32]. Under sodium free conditions, intracellular pH (pHi) changes reflected the H^+, K^+ ATPase activity, since the other sodium-dependent hydrogen transporters were inactive.

After establishing that antibiotics affect transporter activity, we turned to nitric oxide (NO) as a putative mechanism. The NO pathway plays a significant role in intestinal fluid secretion and absorption [33–35]. NO is endogenously synthesized by nitric oxide synthase from the precursors arginine, oxygen, and NADPH [36]. Previously, it has been shown that constitutive NO inhibits NHE3 exchange in enterocytes [37]. Addition of N-nitro l-arginine methyl ester (L-NAME), a NO synthase inhibitor, relieved this NO-induced inhibition (Fig. 1a, b; [37]). Based on this finding, we postulated that NO and its precursors may affect the other H^+ transporter, H^+, K^+ ATPase, in the setting of acute antibiotic exposure.

Materials and Methods

Isolation of colonic tissue

Colonic tissue was obtained from male Sprague-Dawley rats (average weight 250 grams), which were housed in a light-cycled environment with climate and humidity control. All rat handling and experiments were done with adherence to the Institutional Animal Care and Use Committee at Yale University. Rats were fasted overnight with access to water and were euthanized with an overdose of inhaled isoflurane anesthesia prior to surgery. A midline laparotomy was performed and colon between the anorectal junction and splenic flexure was excised. The distal half of the excised descending colon was flushed and longitudinally cut into segments and placed in ethylenediaminetetraacetic acid (EDTA), a calcium-chelation solution, to separate crypts from the basement membrane, as previously described [31, 38]. After incubation, the tissue was centrifuged for 1 minute at 1000 rpm and the pellet was

diluted with cold HEPES-buffered Ringer solution (HBRS, Table 1). The solution was re-centrifuged for an additional minute at 1000 rpm and the subsequent pellet was diluted in additional HBRS.

Fluorescent Dye Loading

The crypts were transferred to a coverslip coated with Cell-Tak adhesive and placed in a thermostatic perfusion chamber at 37°C. The crypt cells were then incubated with HBRS containing 10 μM of a pH sensitive dye, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM, Santa Cruz Biotechnology) over 10 minutes. After, the crypts were bathed with fresh HBRS for 5 minutes to remove residual dye prior to imaging.

Imaging

Following incubation, the dye-loaded crypts in the perfusion chamber were placed on an inverted microscope (Olympus IX70), which was connected to a digital imaging system. The tubing system was arranged so that crypts were superperfused with the solution of interest without interruption, at a constant rate. Within an isolated crypt, five to twelve single regions of interest (ROIs) per colonic crypt and one background ROI were marked on the transferred image (Fig. 1). The midportion columnar cells of the crypts were chosen, excluding the surface epithelial and basal progenitor cells. Throughout the experiment, the crypts were stimulated at wavelengths specific to BCECF-AM, 490 +/− 10 nm and 440 +/− 10 nm, while the emission was measured at 535 +/− 10 nm every 10 seconds. The ratio of dual excitation/single emission was calculated and converted to a pH value, using the high K^+/nigericin calibration technique, previously described [31, 32].

Ammonium prepulse technique

The colonic crypt cells were sequentially perfused with five solutions: 20mM NH_4Cl solution for alkalization, a sodium-free HBRS for rapid acidification, an 8mM Na^+ HBRS for Na^+-dependent pH recovery, and a High-K^+ solution for pH calibration (Table 1). The ammonium prepulse technique was performed in control crypts (n = 7) and in experimental conditions where a drug or combination of drugs was dissolved in each perfusate solution over the course of the experiment: arginine (n = 9 crypts), penicillin G (n=9 crypts), penG + Arginine (n = 17 crypts), pen G + L-NAME (n=7 crypts). In total, aggregate data was collected from 17 Sprague Dawley rats and crypt pHi measurements came from separate rats in each experimental group. To isolate the effects of penG on HKA2, the colonic crypts were incubated with penG and exposed to a sodiumfree environment to temporarily inhibit Na^+-dependent H^+ transporters [38, 39]. The pH_i recovery during the sodium-free phase reflects the actions of the non-Na^+ colonic hydrogen transporters, i.e. the H^+, K^+ ATPase.

Data Acquisition and Analysis

Statistical analysis of data was carried out with GraphPad Prism 8.0 software. To confirm that our data was normally distributed, we performed a Shapiro Wilke test. For comparison of the different mean pH_i recovery rates (pH_i/min), a Tukey's multiple comparisons test was performed as part of an ANOVA. The slope rates were all taken at the same starting pH_i

to eliminate potential changes in intracellular buffering power. The statistical significance is set at a p-value of < 0.05. The results are depicted as mean ± standard error of the mean (SEM).

Drugs

The following drugs were obtained from Sigma-Aldrich Co. and added to solutions in the concentrations listed: 5mM Penicillin G ($C_{16}H_{17}N_2NaO_4S$, CAS Number 69–57-8) and 30μM of L-NAME ($C_7H_{15}N_5O_4 \cdot HCl$, CAS Number: 51298–62-5). Arginine ($C_6H_{14}N_4O_2$) was purchased from MP biomedicals (CAS Number 74–79-3) and prepared at 10mM. All drugs were dissolved in HBRS.

Results

Assessment of rat colonic, H⁺, K⁺ ATPase activity

The degree of H^+, K^+ ATPase activity was demonstrated in colonic crypts by inducing an acid load and monitoring pH_i recovery. After loading with BCECF-AM dye, crypts were exposed to an ammonium prepulse followed by a sodium-free HBRS, which acidified the cells. The pH_i recovery that occurred in this phase represented activity by a sodium-independent H^+ transporter, i.e. H^+, K^+ ATPase. With addition of Na^+ HBRS, the NHE transporters were reactivated and additional proton extrusion occurred. Fig. 2a, b demonstrates the pH_i profiles of individual colonic crypts with and without 5 mM penG. Visually, you can see a significantly steeper slope in the pH_i profile of a crypt perfused with 5 mM penG.

Baseline pH$_i$ recovery rates in 0-Na⁺ control colonic crypts

Control crypts do not show a baseline constitutive H^+, K^+ ATPase activity. The mean rate of pH_i recovery in control colonic crypts perfused under sodium-free conditions was -0.00081 ± 0.0002 pH_i/min (Fig. 2).

PenG increases pH$_i$ recovery rates in 0-Na⁺ colonic crypts, indicating H⁺, K⁺ ATPase activation

When 5mM of penG was added to all solutions perfusing the colonic crypts, there was a positive, sodium-independent pH_i recovery during the 0-Na^+ phase. The mean 0-Na^+ rate of pH_i recovery in colonic crypts perfused with penG alone was significantly higher than in control experiments (Table 2, Fig. 2).

Arginine minimally increases pH$_i$ recovery rates in 0-Na⁺ colonic crypts

When 10mM of arginine was added to all solutions perfusing the colonic crypts, there was a positive sodium-independent pH_i recovery during the 0-Na^+ phase (Fig. 2). The mean 0-Na^+ rate of pH_i recovery in colonic crypts perfused with arginine alone was higher than in control experiments, but not to a statistically significant extent (Table 2, Fig. 2). Also, the pH_i recovery in isolated penG perfusates was higher than isolated arginine perfusion alone, and it just approached significance (Table 2).

Co-perfusion of arginine and penG maximizes pH_i recovery rates

The addition of 10mM arginine and 5mM penG to all solutions led to the greatest significant increase in pH_i recovery during the 0-Na^+ phase (Fig. 2). When compared with controls, this difference was statistically significant (Table 2, Fig. 2). When compared with isolated arginine perfusion, the co-perfusion of arginine + penG also led to statistically significant increase in pH_i (Table 2). However, there was no statistically significant difference in pH_i between crypts perfused with penG alone versus co-perfusion of arginine + penG (Table 2).

L-NAME, a NOS inhibitor, decreases pH_i recovery rates in crypts exposed to penG +/– arginine

Addition of 5mM penG and 30μM L-NAME to the crypt perfusate resulted in a blunted pH_i recovery rate of 0.002411 ± 0.000330 pH_i/min, which was significantly lower than pH_i recovery rates in co-perfused crypts (p 0.0058, Table 2). There was no statistically significant difference in mean pH_i recovery rates between colonic crypts with penG + L-NAME versus control, arginine alone, or penG alone (p 0.5311, p 0.9457, p 0.3024, Table 2).

Discussion

The colonic H^+, K^+ ATPase is a member of the P-type of ATPases, though its role in in K^+ homeostasis is underappreciated [11, 21, 25]. Under the current paradigm, potassium is largely absorbed in the small intestine and secreted in the colon in varying amounts to titrate the final fecal potassium concentration. The potassium handling by colonocytes involves potassium efflux through the apical ENaC and BK channels [27]. On the basolateral side, the Na^+, K^+ ATPase and Na^+-K^+–$2Cl^-$ channel further contribute to potassium shifts. In fluid-constricted states, elevated aldosterone leads to increased HKA2 expression. This hormone results in potassium wasting in order to spare NaCl, and simultaneously upregulates H^+, K^+ ATPase, perhaps as a mechanism of potassium salvage.

This study evaluated whether antibiotics have a non-genomic, non-microbial effect on fluid and electrolyte homeostasis. PenG was selected, due to the fluid modulating effect it was found to have in perfusion analysis on rat colonic segments [30]. Arginine was perfused in isolation and in addition to penG to unveil a potential pathway involving NO. Previously, it was established that NO inhibits NHE3 activity in intestinal villi, though the effect on the colon has not yet been studied.

An established ammonium prepulse technique was implemented to measure the pH_i changes in colonic crypt under sodium-free conditions. The mean change in pH_i was a surrogate measure of the degree of activity of the H^+, K^+ ATPase, since Na/H exchange was inactivated in the absence of sodium.

Under physiological conditions, there is minimal constitutive activity of the H^+, K^+ ATPase. This result was expected given that the majority of potassium absorption occurs in the small intestine, under physiological conditions. We found that the H^+, K^+ ATPase activity increased progressively when colonic crypts were incubated with arginine alone, penG alone, and arginine and penG, respectively (Fig. 3). This study's findings suggest that there

is a significant NO-dependent, penG-sensitive activation of the H^+, K^+ ATPase, as shown in the schematic (Fig. 4a).

When the NO pathway is inhibited with L-NAME, a non-specific NO synthase inhibitor, there is a significant decline in H^+, K^+ ATPase activity (Fig. 4b). The addition of L-NAME to the penG perfusate does not completely abolish H^+, K^+ ATPase activity. This could suggest that there is still a component of H^+, K^+ ATPase activity that is not dependent on the NO pathway. Alternatively, residual HKA2 activity could be due to incomplete blockade of the NO. In the future, various nitric oxide probes can be used to evaluate whether a specific nitric oxide synthase isoform is responsible for HKA2 activation.

Previously, it has been postulated that antibiotics modify the microbiome, leading to downstream effects such as diarrhea [40, 41]. This study demonstrates for the first time that antibiotics directly exert effects at the level of the host colonic transporter. This may have important implications regarding how to mitigate diarrheal fluid loss. On the one hand, antibiotics are associated with diarrhea due to changes in the host microbiome. With these results, however, antibiotics may simultaneously trigger a compensatory host response to spare electrolytes, manifested through the activation of colonic transporters.

To further evaluate the mechanism by which penG upregulates the H^+, K^+ ATPase, this study turned to NO as a putative mediator. NO is formed from L-arginine by NO synthases [42] localized throughout the myenteric and submucosal neurons of the alimentary tract [43]. It facilitates water and electrolyte handling, depending on whether the condition is physiological or pathophysiological. Interestingly, NO has been found to have simultaneous pro-absorptive and pro-secretory effects that may vary between species and under different conditions within species [44]. Here, we determined that the addition of NO precursors stimulated rat colonic H^+, K^+ ATPase under acute penG exposure. Penicillin activates NO production or prevents destruction of NO, which results in elevated levels. Endothelial nitric oxide synthase can then release nitric oxide into the colonic lumen, which can thereby be converted into other more stable nitrogen compounds. From the present data it can be assumed, that penicillin directly interacts with the α- and/or β-subunits of the H^+, K^+ ATPase, since the effect occurs immediately after the antibiotic administration and a transcriptional upregulation of the protein is unlikely to occur within the given time frame. Alternatively, a stimulated translocation and incorporation of preformed H^+, K^+-ATPase containing vesicles to the cell membrane might occur as known for the gastric H^+, K^+-ATPase bearing tubulovesicles in the stomach.

This finding supports the body of literature highlighting NO's absorptive capacity, because H^+, K^+ ATPase activation can lead to passive water absorption. Of interest, it is only when the colonic crypts are co-perfused with penG and arginine that there is an appreciable increase in H^+, K^+ ATPase activity. When arginine was perfused in the colonic crypts alone, there was a mild, statistically insignificant increase in the H^+, K^+ ATPase activity. Overall, further studies are needed to determine how penicillin mobilizes colonocyte nitric oxide.

Potential confounders should be kept in mind when reviewing this study. A cell is a dynamic entity with both ion fluxes that are dependent upon a variety of transporters and channels. In

the present study, we investigated the influence of penicillin family members on the ability of colonic crypt cells to recover their intracellular pH (pH_i) after cellular acid load under Na^+-free conditions, i.e. when the contribution of Na^+-dependent H^+-extruding mechanisms, especially Na^+/H^+-exchangers (NHEs), is annihilated. However, under physiologic conditions, it is possible that basolateral transporters may also impact activity of apical ion handling. Nitric oxide can also affect activity of cellular transporters beyond H^+, K^+ ATPase. Nitric oxide inhibits Na^+,K^+-ATPase in the thick ascending limb of the kidney as well as in intestinal epithelial cells [45]. Na/K/Cl cotransporter activity was also found to be inhibited by NO [46]. To better simulate physiologic conditions, the role of antibiotics on colonic transporters can be evaluated in-vivo or through perfusion of explanated rat intestine placed on an ex-vivo intestinal perfusion device, as described previously [47, 48]. This latter technique would allow us to determine the effect of penicillin on the native colonic microbiome, which was removed during crypt isolation methods.

Overall, we found a NO-sensitive, penG-dependent activation of the colonic H^+, K^+ ATPase. The full capacity of this transporter was only found when the Na/H exchanger was inactivated and when the cells were co-perfused with an antibiotic and a small molecule mediator. Hypokalemic patients may benefit from colonic enema treatments containing penicillin and arginine supplementation as a means to activate the colonic HKA2. Our findings may shed new light on how to reduce fecal potassium loss found in various intestinal diseases.

Acknowledgements

Abbreviations

Arg	Arginine
HBRS	HEPES-buffered Ringers solution
HKA2	H^+,K^+ ATPase isoform 2 or colonic isoform
L-NAME	N-nitro l-arginine methyl ester
NMDG	N-Methyl-D-Glucamine
NO	nitric oxide
penG	penicillin G
pH_i	intracellular pH

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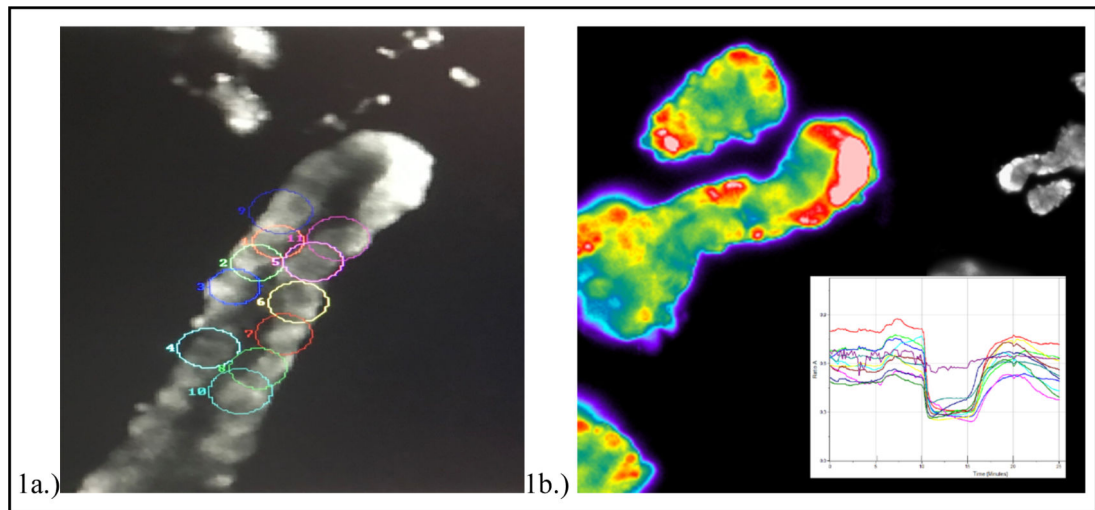
Fig. 1.
1a.) Sample rat colonic crypt with selected regions of interest (ROI) to monitor intracellular pH. 1b.) Pseudocolor image of colonic crypts with filter to visualize local pH values based on fluorescence intensities. Lower right corner is a tracing of multiple cells during and following an NH$_4$Cl prepulse. The red values are the more basic pH values, and the green values are the more acidic.
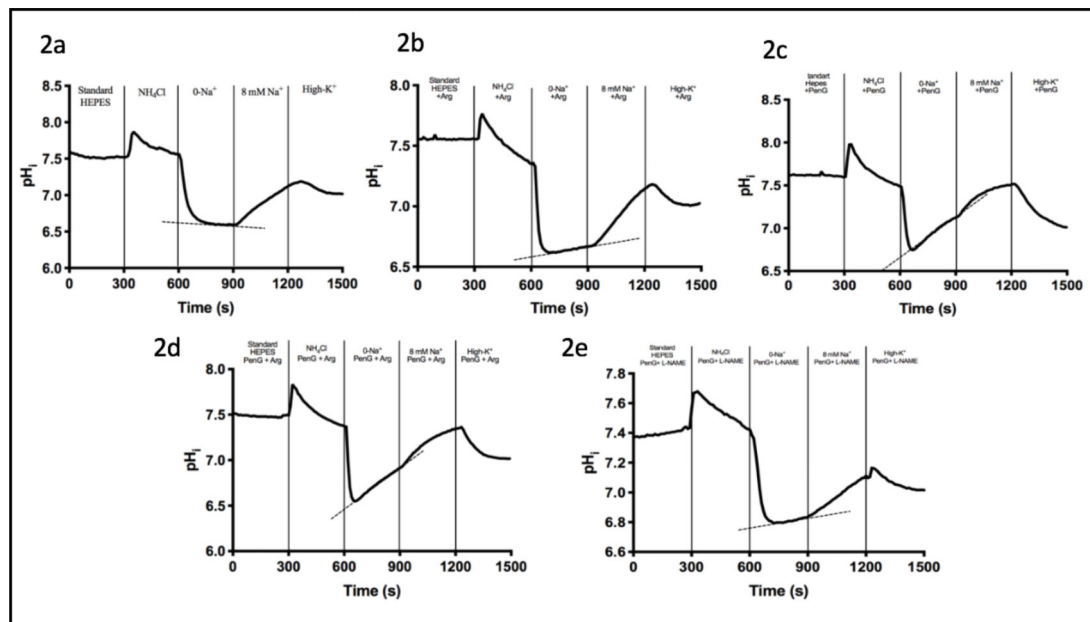
Fig. 2.
Sample pH$_i$ profiles of individual colonic crypts, using the ammonium chloride prepulse technique. Original tracing of pHi changes of a single colonic crypt during a particular experiment. The mean pH$_i$ recovery rate of the crypt during the 0-Na$^+$ phase is quantified and represented by the dashed line. Sample tracings of pH$_i$ changes of a single colonic crypt is shown under all five conditions: control (1a); arginine (1b); penicillin (1c); penicillin + arginine (1d); penicillin + L-NAME (1e). The sequence of solutions under each condition is as follows: i.) Standard HEPES ii.) NH$_4$Cl, iii.) 0-Na$^+$ iv.) 8 mM Na$^+$ v.) High-K$^+$.
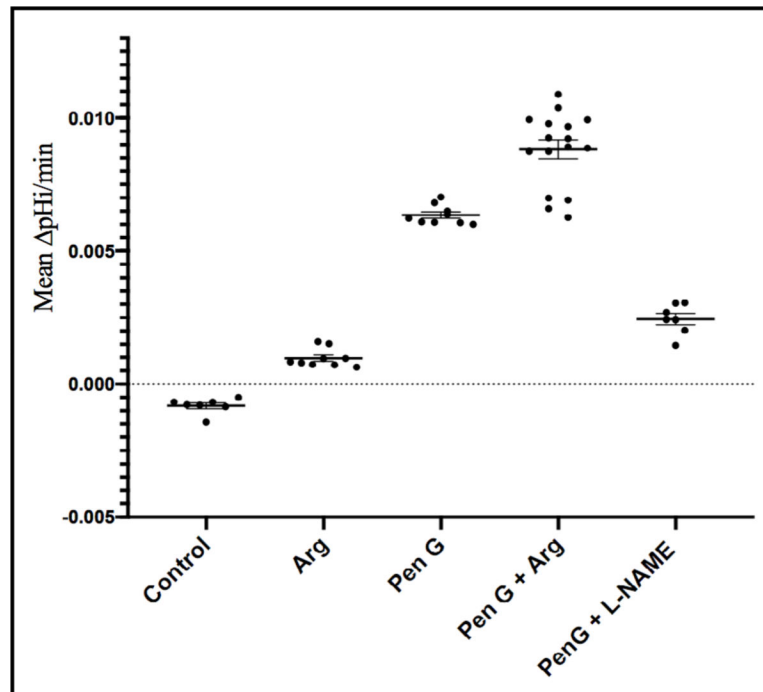
Fig. 3.

pH$_i$ amongst groups during the 0-Na$^+$ phase of the ammonium prepulse technique. The highest ΔpHi is seen in colonocytes perfused with penG + arginine, followed by penG alone. A higher ΔpHi represents a greater degree of activation of the H$^+$, K$^+$ ATPase. The number of rats per group are as follows: Control, n=7; Pen G, n=9; Arginine, n=9; PenG + Arginine, n = 16; PenG + L-NAME, n = 7.
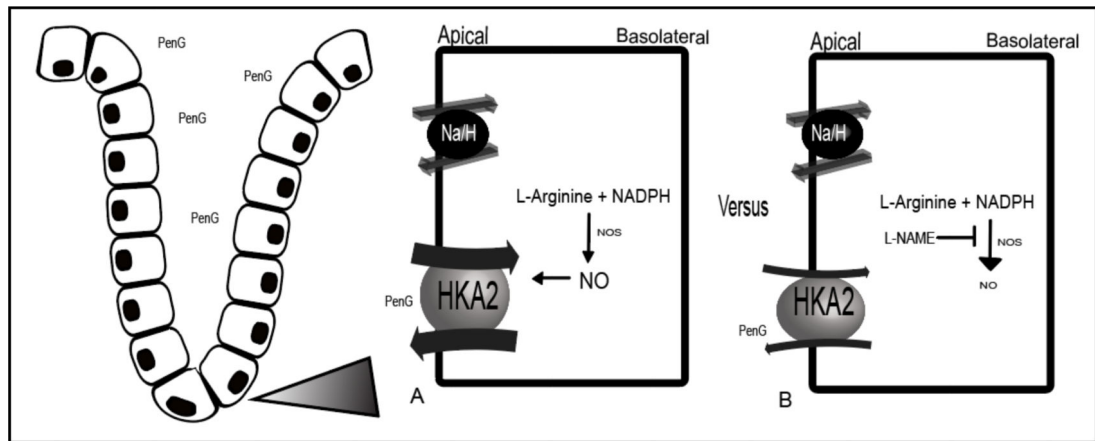
Fig. 4.
Schematic of NO-dependent, penicillin G activation of the H^+, K^+ ATPase in colonic crypts.
4a.) Maximum activation of the H^+, K^+ ATPase occurs when colonic crypts are co-perfused with both arginine and penG. 4b.) When L-NAME is added to the perfusate, nitric oxide synthesis is inhibited. penG still activates the H^+, K^+ ATPase, reflecting a NO-independent, penG-sensitive activation of the transporter.

Table 1.

Composition of Solutions[1].

Solution		Chemical composition
1	HBRS[2]	117 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM Glucose, 32.2 mM HEPES
2	0 Na+ HBRS	132.8 mM NMDG, 5 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM Glucose, 32.2 mM HEPES
3	NH_4Cl HBRS	87 mM NaCl, 30 mM NH_4Cl, 5 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM Glucose, 32.2 mM HEPES
4	8 mM Na+ HBRS	8 mM NaCl, 107 mM NMDG, 5 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM Glucose, 32.2 mM HEPES
5	High K+ Calibration	32.8 mM NMDG, 105 mM KCl, 1.2 mM Mg $MgSO_4$, 1 mM $CaCl_2$, 5 mM Mannitol, 10 mM Glucose, 32.2 mM HEPES

[1] All Solutions used in the experiments were titrated to an osmolarity of 300 +/− 3 mosm and made at 37°C, except for cold and room-temperature HBRS which were at 4°C and 21°C, respectively.

[2] HBRS: HEPES-buffered Ringer solution; NMDG: N-Methyl-D-Glucamine

Table 2.

Mean pHi/min in 0-Na$^+$ in various experimental groups.

	Experimental Group[*]	Experimental Group	Adjusted P values[**]
1	control	pen G	0.0067
2	control	arginine	0.8901
3	arginine	penG	0.0455
4	control	penG + arginine	<0.0001
5	arginine	penG + arginine	0.0001
6	penG	penG + arginine	0.4967
7	penG + arginine	penG + L-NAME	0.0058
8	control	penG + L-NAME	0.5311
9	arginine	penG + L-NAME	0.9459
	penG	penG + L-NAME	0.3024

[*] The ordering of the experimental groups are based on how they appear in the text.

[**] P values were attained through ANOVA and adjusted with Tukey's Multiple Comparisons Test