Benzamil-mediated urine alkalization is caused by the inhibition of \(H^+, K^+\) ATPases

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Abstract:
ENaC blockers elicit acute and substantial increases of urinary pH. The underlying mechanism remains to be understood. Here we evaluate if the benzamil-induced urine alkalization is mediated by an acute reduction in H⁺ secretion via the renal H⁺, K⁺-ATPases (HKA). Experiments were performed in vivo on double HKA knock-out and WT mice. Alterations in dietary K⁺ intake were used to change renal H⁺, K⁺-ATPase and ENaC activity. The acute effects of benzamil (0.2µg/g BW, sufficient to block ENaC) on urine flow rate, urinary electrolyte and acid excretion were monitored in anesthetized, bladder-catheterized animals. We observed that benzamil acutely increased urinary pH (Δ pH: 0.33±0.07) and reduced NH₄⁺ and TA excretion, and that these effects were distinctly enhanced in animals fed a low K⁺ diet (Δ pH: 0.74±0.12), a condition when ENaC activity is low. In contrast, benzamil did not affect urine acid excretion in animals kept on a high K⁺ diet (i.e. during high ENaC activity). Thus, the urine alkalization appeared completely uncoupled from ENaC function. The absence of benzamil-induced urinary alkalization in HKA double knock out mice confirms direct involvement of these enzymes. The inhibitory effect of benzamil is also shown in vitro for the pig α1 isoform of the HKA. These results suggest a revised explanation of the benzamil effect on renal acid-base excretion. Considering the conditions used here we suggest that it is caused by a direct inhibition of the HKAs in the collecting duct and not by the inhibition of ENaC function.

New & Noteworthy
Bolus application of ENaC blockers causes marked and acute increases of urine pH. We provide evidence that the underlying mechanism involves direct inhibition of the H⁺/K⁺ pump in the collecting duct. This could provide a fundamental revision of the previously assumed mechanism that suggested a key role of ENaC inhibition in this response.

Keywords: Benzamil, acid excretion, ASDN
**Introduction:**

Diuretics commonly elicit acute and marked changes in urinary acid-base excretion. It is well known that loop diuretics or ENaC blockers trigger either a marked acute urinary acidification (7, 12, 22) or alkalization (2, 7) respectively.

ENaC-mediated Na\(^+\) transport is electrogenic and the primary cause of the lumen-negative transepithelial voltage present in ENaC expressing epithelia. Thus, when ENaC is activity is changed e.g. during ENaC blockage or physiological up- or downregulation, it directly influences the transepithelial voltage. An ENaC-dependent change of the transepithelial voltage could directly affect the driving force of other electrogenic electrolyte transport processes. This includes H\(^+\) secretion mediated by the vacuolar H\(^+\)-ATPase (V-ATPase), located to the α-intercalated cells (α-IC) in the aldosterone-sensitive distal part of the nephron (ASDN) (2, 34). Several epithelia similar to the ASDN feature electrogenic Na\(^+\) absorption and active H\(^+\) secretion, e.g. frog skin and turtle bladder (3, 14, 15). Both transport processes are voltage-dependent, i.e. can be modulated experimentally by an externally applied voltage (15). In open circuit Ussing chamber experiments, luminal amiloride inhibits both Na\(^+\) transport and active H\(^+\) secretion (3, 14). Importantly, the amiloride-induced inhibition of H\(^+\) secretion is not mediated via direct inhibition of the active H\(^+\) pump but indirectly by reducing the transepithelial voltage (15).

Along the ASDN, the lumen-negative transepithelial potential difference increases (37). This lumen-negative voltage is primarily caused by electrogenic Na\(^+\) absorption via the epithelial Na\(^+\) channel (ENaC) located to the principal cells (PC). A diffusional voltage component due to lower luminal Cl\(^-\) concentrations and an anion-selective paracellular shunt pathway could provide an additional input to this lumen-negativity. Loop diuretics, such as furosemide, inhibit Na\(^+\) absorption by blocking the Na\(^+\)/K\(^+\)/2Cl\(^-\) transporter (NKCC2) in the thick ascending limb of the loop of Henle (TAL). Thus, the
amount of Na\(^+\) reaching the ASDN increases following furosemide application. This would lead to an increased uptake of Na\(^+\) via ENaC, an increase in the lumen-negative transepithelial voltage and a subsequent increase in V-ATPase-mediated H\(^+\) secretion causing urine acidification. Conversely, benzamil-dependent ENaC inhibition diminishes the transepithelial potential difference (2) and thus, should decrease V-ATPase-mediated H\(^+\) secretion (19). However, older results (26) and also newer experimental data have questioned, whether changes in transepithelial voltage is a satisfactory explanation for the pronounced acute urine acidification by furosemide. A previous study from our group showed that acute furosemide-induced urinary acidification is mediated by an acute and strongly increased function of the apical Na\(^+\)/H\(^+\) exchanger 3 (NHE3), located to the TAL(12). In a follow-up study, we did not find any acute urinary pH changes after the administration of the thiazide diuretic hydrochlorothiazide (HCT) despite increased Na\(^+\) delivery to the CNT/CD. This was found both under control conditions and conditions with molecular upregulation and functional activation of ENaC(4). In other words, providing more substrate for electrogenic Na\(^+\) absorption did not trigger increased tubular H\(^+\) secretion as would have been expected, if ENaC-dependent voltage changes drive large urine pH changes in this in vivo situation. These studies, however, do not explain how the inhibition of ENaC with e.g. amiloride or benzamil triggers very sizeable and acute urine alkalizations as has been reported consistently in animal and human studies (2, 7). Since the commonly used explanation appears not sufficient, we must find another way to understand the urine alkalization following administration of ENaC blockers.

In this project, we address the mechanism of benzamil-induced urine alkalization and its relation to the H\(^+\), K\(^+\)-ATPase (HKA). This pump is expressed in α-IC apical membrane in two isoforms, the gastric (HKα1) and colonic (HKα2), and mediates K\(^+\) absorption and H\(^+\) secretion (18). We provide evidence that benzamil induces marked urine alkalization by direct inhibition of H\(^+\) secretion of the CD HKAs.
Methods:

Animals and Diets:

All animal handling was in accordance with animal welfare regulations (Animal experiment licence from the Danish Animal Welfare Regulation Authority: 2016-15-0201-01129). In a first experimental series, 8-10 week old male C57/Bl6J mice, purchased from Janvier, France were used. As a control diet Altromin 1310 (Altromin Spezialfutter GmbH & Co. KG, Germany) with a 0.9 % K⁺ content and a 0.2% Na⁺ content was used. To induce an upregulation of ENaC and a down regulation of renal HKA, mice were fed a high K⁺ diet (5%) for 4 days.(16) The diet was made by adding KCl to a ssniff EF R/M control diet (0.97 % K⁺, 0.2% Na⁺, ssniff, Spezialdiäten GmbH, Germany). Conversely, to induce a downregulation of ENaC and an upregulation of renal HKA mice were fed a pre-made low K⁺ diet (<0.03% K⁺, Altromin C1037) for 3 days. Additionally, mice were fed a low K⁺ high Na⁺ for 4 days. The diet was made by addition of NaCl to a low K⁺ diet (<0.03% K⁺, Altromin C1037) with a final NaCl content of 2%.

Renal HKA dKO mice derived from two colonies of mice originally constructed by Gary Shull that disrupted both the ATP4a gene that encodes HKα1 and the ATP12a gene that encodes HKα2 (27, 32) were bred onto a C57Bl6J line for more than ten generations as previously documented (24) with verification of genotype at each generation.

Real-time, in vivo urinary pH measurements

The mice were anesthetized via an intraperitoneal bolus injection of a ketamine (10mg ml⁻¹)/xylazine (1mg ml⁻¹) mix at a dose of (10µl g BW⁻¹). An intravenous catheter was established in one of the animals’ tail veins and anaesthesia was maintained by continuous infusion of a third of the induction
dose per hour applied in 0.9% sterile saline. Thereafter, a custom-made catheter was surgically inserted in the urinary bladder. Micro-pH electrodes (Ø 200, Unisense, Denmark) were placed in the outflow of the catheter and urinary pH was measured continuously every second. To monitor urine flow rate, urine was collected from the tip of the catheter every 5 min. The collected urine was stored at -20° C for further analysis. Benzamil was dissolved in a Ringer’s solution with 0.5% DMSO. In a first experimental series, investigating the benzamil effect under different dietary K⁺ intake all animals received an i.p. bolus injection of benzamil (0.2 µg/g BW). In the second experimental series, renal HKA dKO and wild-type mice received benzamil. A subset of renal HKA dKO animals received a vehicle solution (isotonic ringer’ solution with 0.5% DMSO) as a control group.

**Flame photometry**

Urinary [Na⁺] and [K⁺] were measured using a model 420 Flame Photometer (Sherwood Scientific, UK). From the urine flow rate data, absolute urinary Na⁺ and K⁺ excretion rates were determined.

**Western blots:**

Tissue harvesting for western blots was performed as described in Larsen et. al. (23). Briefly, western blotting was done on the supernatant of ½ kidneys, processed in lysis buffer in a tissuelyser in 30 sec. (Qiagen) and spun for 15 min. (1000G).

Protein concentrations were measured using Pierce™ BCA Protein Assay Kit. All samples were run on criterion™ TGX™ Stain-free 4-15% gels (Bio-Rad, 5678085). 10 µg of total protein was loaded to each sample well. After loading, gels were activated using a Azure c600 imaging system (Azure Biosystems) allowing normalization of the density signals of the protein of interest to the total amount of protein loaded (Suppl. Fig. 1: [https://doi.org/10.6084/m9.figshare.13341320.v1](https://doi.org/10.6084/m9.figshare.13341320.v1)). Membranes were developed using clarity™ Western ECL substrate (Bio-Rad) in an ImageQuant LAS 4000 mini (GE Healthcare).
Healthcare Life Science). All images were analyzed using Image Studio™ Lite (Li-Cor). The used antibody information is listed below.

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**Measurement of urine NH$_4^+$ and titratable acid excretion**

The collected urine from each mouse was pooled into a pre benzamil sample and a post benzamil sample and analyzed for NH$_4^+$ and titratable acids. Urine NH$_4^+$ concentration was measured using an ammonia ion-selective electrode as previously done (8). Urine titratable acid concentration was measured by titration using the method by Chan (10) adapted to small volume samples as previously done (8). NH$_4^+$ and titratable acid excretion rates were calculated by multiplying with the actual urine flow rate in each time interval.

**Measurement of gastric H$^+$, K$^+$-ATPase activity**

Pig gastric H$^+$, K$^+$-ATPase-enriched membrane vesicles were prepared as described earlier (1). The preparation was essentially free from Na$^+$, K$^+$-ATPase as shown by the absence of ouabain-sensitive activity. Maximal activity of the given preparation under optimal conditions at 37 °C was 250 µmol P$_i$/mg protein per hour. H$^+$, K$^+$-ATPase preparation was permeabilized with a channel-forming peptide alamethicin dissolved in 50 % v/v ethanol at the ratio alamethicin/protein 0.6/1 by 20 min incubation in the media containing EDTA 0.2 mM, MgCl$_2$ 5 mM, varying KCl (2,10,20 mM) and a buffer mixture of
His 20 mM + Tris 20 mM (covering range of pH 6.0, 7.0, 8.0). The addition of benzamil at varying concentrations was followed by a second 5 min pre-incubation at 37 °C, prior the reaction was initiated by 3 mM ATP. After 5 min, the hydrolytic reaction was terminated and the amount of P_i was determined according to Baginski (5). Each measurement was performed in triplicate, the data are presented as mean value ± SD.

Measurement of pig kidney Na^+, K^+-ATPase activity

Na^+, K^+-ATPase preparation was isolated from pig kidney according to Klodos et al. (21). Specific Na^+, K^+-ATPase activity, i.e. the difference in amount of phosphate released in the absence and presence of 1 mM ouabain, was 1800 µmol P_i/mg protein per hour at 37 °C. Inhibitory effect of benzamil on the enzyme was evaluated in a solution containing 30 mM Histidine pH 7.4, 130 mM NaCl, 20 mM KCl, 4 mM MgCl_2 and 3 mM ATP in the way described above for experiments with H^+, K^+-ATPase. Each measurement was performed in triplicate, the data are presented as mean value ± SD.

Analysis and statistics

GraphPad Prism version 8.4.2 (GraphPad Software, USA) was used to perform statistical analysis. The distribution of data was evaluated by the generation of QQ plots. When the data distribution deviated from Gaussian function, log-transformation was applied. Alternatively, non-parametric tests were employed. Paired/unpaired t-tests were performed within/between the groups, respectively. Mann-Whitney tests were used for unpaired data deviating from normality. Comparisons of multiple groups were performed by one-way ANOVA with Bonferroni correction. Figure legends detail statistical analysis for each case. Data are shown as mean ± SEM or SD as indicated in figure legends. All statistical tests were two-sided with a minimal accepted significance of p = 0.05.
Results:

The acute benzamil-induced urine alkalizing effect in high K\(^+\), control and low K\(^+\) diet treated mice.

First, using a bolus dose of benzamil known to fully block ENaC (20), we studied the effect of benzamil on urine pH and excretion of NH\(_4\)\(^+\) and TA. We found that low K\(^+\) diet treated animals have a markedly increased urine acidity with a baseline pH near 5.9 as compared to a urine pH near 6.8 in control K\(^+\) diet fed animals and a pH urine value near 7 in mice fed a high K\(^+\) diet (Fig. 1A,C). Remarkably, benzamil induced an acute and very large urine alkalization in low K\(^+\) diet fed mice paralleled by a large drop in NH\(_4\)\(^+\) and TA excretion (Fig. 1A,C-F). (see non-compiled NH\(_4\)\(^+\) and TA urine data in suppl. fig. 2: https://doi.org/10.6084/m9.figshare.13341335.v1) In control K\(^+\) diet fed mice, the benzamil-induced urine alkalization was still present but markedly attenuated as was the drop in NH\(_4\)\(^+\) and TA excretion (Fig. 1A, C-F). In animals on high K\(^+\) diet, benzamil showed no acute effects on urine acid excretion. Fig. 1B depicts the associated benzamil-induced acute reduction of urine [K\(^+\)] in the three diet groups. These data confirm previous observation of acute urine alkalization induced by benzamil or amiloride (2, 7). Moreover, they reveal a clear correlation between the amplitude of benzamil-induced effects and dietary K\(^+\) intake. Thus, animals on a low K\(^+\) diet produce acidic urine while benzamil the effect on pH is strongly amplified.

The acute benzamil-induced effects on urine K\(^+\), Na\(^+\) and volume excretion in high K\(^+\), control and low K\(^+\) diet treated mice.

We next studied the effect of benzamil on urine K\(^+\), Na\(^+\) and volume excretion under the three K\(^+\) diet conditions (Fig. 2A-F). As expected, benzamil caused a marked and prompt reduction of urine K\(^+\) excretion in control and high K\(^+\) diet conditions. In mice fed a low K\(^+\) diet, baseline urine K\(^+\) excretion was very low and not further reduced by benzamil. Urine Na\(^+\) excretion was increased rapidly and
reached similar values in all K⁺ diet groups. Benzamil showed no effect on urine flow in any of the three experimental groups. These results confirm the efficacy of benzamil to increase urinary Na⁺ excretion and reduce urinary K⁺ excretion as would be expected from a drug that blocks ENaC.

Regulation of functional ENaC protein expression in high K⁺, control and low K⁺ diet treated mice

ENaC function and expression is a key element to drive K⁺ secretion in the ASDN and is known to be markedly regulated by dietary K⁺ intake. Mice on a high K⁺ diet increased total α-ENaC (90 kDa) expression and mice on a low K⁺ diet reduced total α-ENaC protein expression. In addition, total γ-ENaC (95kDa) expression was reduced and the cleaved shorter version of γ-ENaC (70 kDa) was increased in the high K⁺ diet group (Fig. 3A,B). Opposite γ-ENaC protein expression results were obtained when mice we fed a low K⁺ diet. These results confirm previous data from the literature (17) and indicate a marked functional upregulation of ENaC following a high K⁺ diet and the reciprocal results on the low K⁺ diet.

Abolished benzamil-induced urinary alkalization in the double H⁺, K⁺-ATPase knock-out mouse.

We then studied mice that were genetically depleted of the two H⁺, K⁺-ATPase (dKO) isoforms to determine whether the benzamil-induced urine alkalization could be induced (Fig. 4A-F). Baseline urinary pH was similar between the different groups. As expected, benzamil acutely increased urinary pH and reduced NH₄⁺ and TA excretion in the wild-type group that was very similar to the results show in Fig. 1. (see non-compiled NH₄⁺ and TA urine data in suppl. fig. 3: https://doi.org/10.6084/m9.figshare.13341347.v1) The key finding of this experimental series is that benzamil neither increased urine pH nor caused a decrease in NH₄⁺ and TA excretion in the dKO
animals. These results imply that H\textsuperscript{+}, K\textsuperscript{+}-ATPase function in the α-ICs of the CD is required to trigger the benzamil-induced urine alkalization.

The acute benzamil-induced effects on urine K\textsuperscript{+}, Na\textsuperscript{+} and volume excretion in double H\textsuperscript{+}, K\textsuperscript{+}-ATPase knock-out mice and their wild-type controls.

Benzamil did not elicit acute changes in urine flow rate of any group (Fig. 5E,F). Baseline Na\textsuperscript{+} excretion was similar in all groups (Fig. 5C). Benzamil significantly increased Na\textsuperscript{+} excretion (Fig. 5C). The benzamil-induced Na\textsuperscript{+} excretion was significantly smaller in the dKO group (Fig. 5C,D) consistent with lower functional ENaC in the dKO group (Fig. 6). Baseline kaliuresis was lower in the dKO animals as compared to wild-types (Fig. 5A) consistent with the K\textsuperscript{+} deplete state in the dKO mice. Benzamil significantly decreased kaliuresis in the wild-type group similar to the effect shown in Fig. 1. In the dKO group, benzamil had no effect on kaliuresis (Fig. 5A,B). In time control experiments in dKO mice kaliuresis increased during the course of the 90 min. experiments (Fig. 5A,B). These results describe the phenotypical difference of resting and benzamil-induced Na\textsuperscript{+} and K\textsuperscript{+} excretion in between dKO mice and their wild-type counterparts.

ENaC protein expression in double H\textsuperscript{+}, K\textsuperscript{+}-ATPase knock-out mice and their wild-type controls.

We compared α- and γ-ENaC protein subunit and their respective cleavage product expression between dKO and wild-type control mice. Double KO mice had lower total α-ENaC (90 kDa) expression and also lower expression of the α-ENaC 34 kDa cleavage product (Fig. 6A). In addition, dKO mice also showed reduced amount of the cleaved 70 kDa part of γ-ENaC. The expression level of total γ-ENaC
(95 kDa) was not different in between the genotypes. These results indicate slight functional down-regulation of active ENaC proteins in dKO mice.

Inhibition of pig gastric H\(^+\), K\(^+\)-ATPase and renal Na\(^+\), K\(^+\)-ATPase by benzamil.

Previous studies have shown that benzamil directly inhibits mouse gastric HKA. Here we measured the effect of benzamil on pig gastric HKA under different pH and extracellular K\(^+\) concentrations (Fig. 7 A-D). We have chosen pH values of 6, 7 and 8 and [K\(^+\)] of 20, 10 and 2 mM to approximate the physiological values in the collecting duct *in vivo*. HKA activity decreased upon benzamil binding under all conditions. The mechanism of inhibition appears to be of a mixed type, where enzyme affinity to K\(^+\) (substrate) decreased in the presence of benzamil (inhibitor) and *vice versa*, while pH had no influence on benzamil affinity. These results reveal that (i) benzamil binding site is different from the ion-binding sites of the enzyme; and (ii) benzamil-enzyme complex retains some ATPase activity. It turned out, that benzamil inhibits also pig renal Na\(^+\), K\(^+\)-ATPase, i.e. it is promiscuous towards P-type ATPases (Fig. 7E).

Discussion:

*Benzamil stimulated urinary alkalization does not correlate with ENaC activity.*

If ENaC dependent voltage changes would be the cause of the urine alkalization related to benzamil treatment, the magnitude of benzamil’s alkalizing effect should correlate with the *a priori* functional ENaC activity. Therefore, in an attempt to alter functional ENaC activity, we have used different dietary regimens. A high K\(^+\) diet induces a marked upregulation of molecular ENaC expression (17) and increases the delivery of Na\(^+\) to the site of ENaC. The latter effect is due to a downregulation of the
more proximally located Na⁺/Cl⁻ cotransporter (NCC) (30, 33) located to the distal convoluted tubule. Opposite effects, namely lowering of ENaC expression and distal Na⁺ delivery, occur under K⁺ restriction (17, 33). Thus, functional ENaC activity is increased and decreased when feeding either a high K⁺ or low K⁺ diet, respectively. We confirm K⁺ diet-dependent regulation of ENaC in our study (Fig. 3). Assuming that functional ENaC activity determines benzamil-induced urinary alkalization, one should expect a greater increase in urinary pH and reduction of NH₄⁺ and TA excretion following benzamil in the high K⁺ diet group as compared to the low K⁺ diet group. However, the findings of the present study are in sharp contrast to this expectation. The benzamil-induced urinary alkalization was greatly enhanced under K⁺ restriction and conversely near absent in mice kept on a high K⁺ diet. During the high K⁺ diet where ENaC function is strongly upregulated, benzamil markedly reduced urine [K⁺] and K⁺ excretion and stimulated Na⁺ excretion. This provides good evidence for a sound in vivo ENaC blocking effect under these conditions. By all likelihood, this must have reduced the lumen-negative voltage in the ASDN but we could not detect any change of NH₄⁺ and TA excretion nor an effect on urine pH by benzamil under these conditions. Thus, the benzamil-induced urinary alkalization markedly dissociated from the functional activity of ENaC.

A low K⁺ diet causes a strong urine acidification and a pronounced upregulation of the benzamil-stimulated urine alkalization

The absence of a correlation between the benzamil effect and functional ENaC activity further substantiates the need for an alternative explanation of its marked alkalizing effect. As mentioned, we studied the alkalizing effect of benzamil under different baseline HKA activity levels. Abundant evidence has documented that dietary K⁺ intake correlates inversely with renal and colonic HKA
expression and activity (18, 31). The benzamil effect was studied under high and low K+ intake, respectively. Baseline urinary pH might be an indirect estimate of whether the applied dietary regimens actually altered baseline renal HKA activity. Indeed, the markedly lower baseline urinary pH in the low K+ diet group is compatible with an enhanced activity of the HKAs under K+ restriction. Possibly, other activated H+ secretory mechanisms could also be in play as suggested by a previous micropuncture work (6). The much larger benzamil-induced urinary alkalization and reduction of NH4+ and TA excretion in the low K+ diet group suggests that the benzamil effect could involve an inhibition of HKA activity.

This was therefore tested more directly by using mice genetically deplete of HKAases. There are two isoforms of the HKA, α1 and α2, respectively. Since both HKA isoforms are expressed in the kidney (18), we chose to use the double HKA knock-out model. The absence of benzamil-induced urinary alkalization in HKA dKO animals supports the interpretation of an HKA-dependent effect. It should be noted that the animal model used here is characterized by an obligate fecal K+ losing phenotype (27). This is reflected in lower baseline urinary [K+] in dKO animals as compared to wild-type animals. Chronic K+ wasting/depletion is associated with decreased ENaC expression and lower aldosterone (see Fig. 3) (36). Indeed, we found a decreased expression of functional ENaC in dKO mice (Fig. 6). This likely explains the presently observed attenuated benzamil effect on Na+ excretion in the dKO group. However, benzamil still elicited a natriuretic and antikaluetic effect in dKO mice. Thus, we conclude that the benzamil-mediated alkalization cannot be seen in the absence of the HKAs.
What is the underlying mechanism leading to the apparent benzamil-inhibited HKA function?

The presented data indicates that the benzamil-induced acute urine alkalization requires the functional presence of HKA function in the collecting duct. The mechanism by which benzamil triggers HKA inhibition is currently not fully defined. Two suggested mechanistic scenarios argue either for a direct HKA inhibition by benzamil or for an indirect inhibitory effect of low tubular [K⁺] caused by ENaC inhibition. Interestingly, amiloride and benzamil were shown to directly inhibit α₁-HKA (29) and also NHE3 activity (25, 28). These findings raise the question whether the alkalizing effect of benzamil is mediated by direct inhibition of these H⁺-secreting mechanisms. We have previously defined the in vivo threshold benzamil dose in mice that is sufficient to fully block ENaC in the CCD (20) and this dose was used in the current paper. In native rodent colon, ENaC is fully inhibited by >1 µM benzamil (9). On these grounds, we assume that the ambient benzamil concentrations in the CCD in our experiments must have reached 1 µM or slightly higher. This argues strongly against an effect of benzamil on NHE3 in any part of the renal tubule as this transporter requires much higher blocker concentrations (IC₅₀ ~100µM) (28). As for the HKA, a concentration of 100µM of amiloride or benzamil was needed to induce a ~65% decrease in α₁ HKA activity (29). The α₂ HKA activity originating from mouse distal colon was not inhibited with amiloride or analogues up to a concentration of 100 µM (29). Our pig gastric HKA experiments demonstrate that ~50 µM benzamil are sufficient to inhibit 50% pump activity (Fig. 7). The benzamil concentration in the cortical CD lumen is assumed to be close to 1 µM or higher. However, the HKA activity localizes to ICs reaching down to the outer medullary collecting duct (OMCD) (11) and thus the dynamic process of urine concentration should have led to higher ambient benzamil concentrations in the lumen of the more distally located OMCD consistent with HKA inhibition. A low K⁺ diet is well established to trigger a marked renal up-regulation of the colonic isoform (α₂ HKA) reportedly accounting for the entire HKA
activity during these conditions (11). This suggests a shift from $\alpha_1$ HKA to $\alpha_2$ HKA function under K$^+$ depletion (13). The here reported benzamil effect is very prominent in mice treated on a low K$^+$ diet suggesting that benzamil actually targets the $\alpha_2$ HKA isoform in our low K$^+$ diet experiments. This does not align with the reported absence of a benzamil effect in isolated mouse colonic HKA (29).

Importantly, the compiled evidence of the pharmacological properties of HKA functions indicates a high degree of species and tissue heterogeneity (11). The structural and functional homology of the two isoforms of the HKA actually makes it likely that benzamil targets both pumps (11). This conclusion is further supported by our results showing that the homologous Na$^+$, K$^+$-ATPase can be inhibited by benzamil, also (Fig. 7E). We further studied the effect of benzamil in mice on a high Na$^+$/low K$^+$ diet to create a condition of suppressed ENaC function and upregulated HKA function. We speculated that under these conditions the ENaC-blocking effect causing reduced urine K$^+$ excretion could become even increase due to the benzamil-induced inhibition of K$^+$ absorption via the HKA. We found that benzamil-induced urine alkalization prevailed (suppl. Fig. 4 https://doi.org/10.6084/m9.figshare.13341365.v1) while no apparent increase of urine K$^+$ excretion was not found under these conditions.

In summary, we suggest that the benzamil-induced urine alkalization effect is likely caused by direct pharmacological inhibition of both the colonic and the gastric HKA. Using the double HKA KO mouse precludes a firmer definition, which HKA isoform is involved in the benzamil effect and further studies using the single HKA KO are desirable.

Inhibition of ENaC with benzamil causes the well-known inhibition of K$^+$ secretion in CD principal cells, which led to the observed marked [K$^+$] lowering in the final urine. The question arises if the ambient luminal [K$^+$] concentrations in the CD after benzamil could reach low enough values causing substrate (luminal K$^+$) scarcity for the HKAs to function improperly. If this were the case, HKA pump
activity would be inhibited as an indirect consequence of luminal K⁺ depletion due to inhibition of
more upstream K⁺ secretion. The K⁺ dependences of the α₁- and α₂-isoforms of murine HKA have been
determined in membrane vesicles and the Kₘ values for [K⁺] of the α₁- and α₂-isoforms were 77µM
and 40µM, respectively (29). A close inspection of this publication indicates that a significant HKA
pump inhibition can be expected at ambient, luminal [K⁺] below 1 mM. In our experiments using low
K⁺ diet treated animals, we measured that benzamil triggered a [K⁺] lowering effect in the final urine to
concentrations near 10 mM. Because the final urine concentration of K⁺ is dependent on the degree of
water reabsorption along the CD, the ambient luminal [K⁺] at the site of HKA must be significantly
lower as compared to that found in the final urine. An estimation of this adjustment factor would
assume an osmolarity of around 100 mosmol/L in the early distal tubule and a final urine osmolarity
around 2000 mosmol/L. Thus, a concentrating factor of ~20 seems reasonable. This would suggest that
the ambient [K⁺] at the site of the HKAs may reach values below 1 mM. Thus, it cannot be excluded
that the in vivo mechanism of benzamil-induced HKA inhibition is initiated by a very low luminal [K⁺]
that reduces HKA pumping activity. However, two distinct observations argue against the latter
mechanism. Firstly, benzamil also triggers a significant alkalinization under control K⁺ diet conditions
were urinary [K⁺] does not fall much below 50 mM. Secondly, a close temporal association between
the benzamil-induced alkalinization and the drop of urine [K⁺] is not visible. A close inspection of the
data in Fig. 1A and B (red curves) indicates that the benzamil-induced alkalinization is prompt and
occurs after only 3 min. post injection. In contrast, the benzamil-induced [K⁺] lowering effect occurs
much more sluggishly becoming obvious first at > 10 min. after benzamil injection. However, the
benzamil-induced reduction of urine [K⁺] may have an important impact on its suggested inhibition of
HKA function. Benzamil is a more effective HKA blocker at lower extracellular [K⁺] (Fig. 7D) and
hence a [K⁺] lowering may elicit synergistic HKA blocking effects.
This paper does not rule out that ENaC function and the physiology of a lumen-negative voltage in the collecting duct can be a driver for increased V-type dependent H^+ secretion as suggested by studies in frog skin or turtle bladder under some conditions. However, in the case of the benzamil-induced urine alkalization, ENaC dependent changes of transepithelial voltage appears unlikely to be the explanation. On the basis of the results presented in this paper, we propose that the benzamil-induced urine alkalization is triggered by a direct inhibition of the HKAs in the collecting duct. This effect is greatly enhanced after a low K^+ diet treatment because tubular HKA activity is markedly upregulated and benzamil affinity for these ATPases is highest at low K^+ concentrations. Fig. 8 provides a graphical view of the suggested mechanism of benzamil-mediated urine alkalization.
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Conflict of interest:
No conflicts of interest are declared by the authors.
References:


Figure legends:

**Figure 1:** Benzamil (0.2µg/g BW) induced *in vivo* effects on urinary pH, urine K⁺ concentration and acid excretion (NH₄⁺ and titratable acid (TA)) under high K⁺ (green, n=10), normal K⁺ (black, n=19) and low diet (red, n=6). **A:** Resting and benzamil-induced urine pH effects in the three diet conditions. **B:** Benzamil-induced urine K⁺ concentration reductions in the three diet conditions. **C and D:** Summary of benzamil induced urine pH effects. **E and F:** Summary of benzamil-induced NH₄⁺ and TA excretion changes. (paired t-tests in C and E. One-way ANOVA followed by Bonferroni’s multiple comparisons test in D and F. **p<0.01; ***p<0.001, **** p<0.0001).

**Figure 2:** Benzamil (0.2µg/g BW) induced effects on, K⁺, Na⁺ and water excretion given at 30 min under high K⁺ (green, n=10), normal K⁺ (black, n=19) and low diet (red, n=6). **A and B:** Benzamil-induced reductions of K⁺ excretion under high K⁺ (green, n=10), normal K⁺ (black, n=19) and low diet (red, n=6). **C and D:** Benzamil-induced increases of Na⁺ excretion under high K⁺ (green, n=10), normal K⁺ (black, n=19) and low diet (red, n=6). **E and F:** Benzamil did not change urine flow in any conditions (high K⁺, green, n=10) (normal K⁺, black, n=19) and (low K⁺, diet red, n=6). (One-way ANOVA followed by Bonferroni’s multiple comparisons test in B, D and F. *p<0.05).

**Figure 3:** Immunoblots showing molecular up-regulation of ENaC in mice subjected to a high K⁺ diet and molecular down-regulation of ENaC in mice subjected to a low K⁺ diet. §: The gel broke during transfer. This part of the full length blot was not included in the statistical analysis. **A:** Full length (90 kDa) and cleaved (~34 kDa) α-ENaC in kidney-lysates from mice subjected to a control, a low K⁺ or a high K⁺ diet. Note that both full length and cleaved α-ENaC expression is markedly higher in kidneys.
from mice on a high K⁺ diet and lower in kidneys from mice on a low K⁺ diet, one-way ANOVA followed by Bonferroni’s multiple comparisons test. **B:** Full length (95 kDa) and cleaved (70 kDa) γ-
ENaC in kidney-lysates from mice subjected to a control, a low K⁺ or a high K⁺ diet. Note that cleaved γ-ENaC expression is markedly higher in kidneys from mice on a high K⁺ diet and lower in kidneys from mice on a low K⁺ diet, one-way ANOVA followed by Bonferroni’s multiple comparisons test. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

**Figure 4:** Benzamil (0.2µg/g BW) induced *in vivo* effects on urinary pH, urine K⁺ concentration and acid excretion (NH₄⁺ and titratable acid=TA) in WT (n=5) and dKO HKA (n=5) mice. **A:** Resting and benzamil-induced urine pH effects in benzamil treated WT (black curve, n=5), benzamil treated dKO HKA (dark blue curve, n=5) and vehicle treated dKO mice (light blue curve, n=4). **B:** Benzamil-induced urine K⁺ concentrations reductions in the three groups. **C and D:** Summary of benzamil induced urine pH effects. **E and F:** Summary of benzamil-induced NH₄⁺ and TA excretion changes. (paired t-tests in C and E, one-way ANOVA followed by Bonferroni’s multiple comparisons test in D and F. *p<0.05; **p<0.01; ***p<0.001, **** p<0.0001).

**Figure 5:** Benzamil (0.2µg/g BW) induced effects on, K⁺, Na⁺ and urine excretion given at 30 min in WT (n=5) and dKO HKA (n=5) mice. **A and B:** Benzamil-induced effects on K⁺ excretion in WT (black curve, n=5) and dKO HKA (dark blue curve, n=5). Also shown are vehicle treated dKO mice (light blue curve, n=4). **C and D:** Benzamil-induced increases of Na⁺ excretion in WT (black curve, n=5) and treated dKO HKA (dark blue curve, n=5) mice. Also shown are results from vehicle treated dKO mice (light blue curve, n=4). **E and F:** Benzamil did not change urine flow in WT (black curve, n=5) or dKO HKA (dark blue curve, n=5) mice. Also shown are the results from vehicle treated dKO
mice (light blue curve, n=4). (one-way ANOVA followed by Bonferroni’s multiple comparisons test in B, D and F. *p<0.05, **p<0.01; ***p<0.001).

Figure 6: Immunoblots showing a modest molecular down-regulation of ENaC in dKO mice compared to their WT littermates. Quantifications show the mean of three duplicate runs of the same samples. A: Full length (90 kDa) and cleaved (~34 kDa) α-ENaC in kidney-lysates from dKO and WT mice. Note that cleaved α-ENaC expression is approximately 30% decreased in dKO mice as compared to WTs. B: Full length (95 kDa) and cleaved (70 kDa) γ-ENaC in kidney-lysates from dKO and WT mice. Note that cleaved γ-ENaC is approximately 20% lower in dKO mice as compared to WTs. (students t-test. *p<0.05).

Figure 7: Inhibition of pig gastric H^+,K^+-ATPase by benzamil. Residual activity was measured after preincubation of the membrane-bound H^+,K^+-ATPase for 5 minutes at pH 6 (A), 7 (B) and 8 (C) at KCl concentrations of 20, 10 and 2 mM. D: IC50 values derived from A-C are shown as a function of the KCl concentration at pH 6, 7 and 8. E: Inhibition of the pig renal Na^+, K^+-ATPase by benzamil. All measurements were performed in triplicate, the data are presented as mean value ± SD.

Figure 8: Schematic graph of the suggested mechanism of benzamil-induced urinary alkalization. A low K^+ diet increases functional HKA activity, causing decreased urinary pH. Functional ENaC activity is decreased during a low K^+ diet. Benzamil inhibits ENaC leading to an increased Na^+ excretion and inhibition of ROMK. Benzamil directly inhibits HKA activity. During a low K^+ diet, benzamil may also indirectly inhibit HKA by desaturation of the pump caused by cessation of K^+ secretion by ROMK during ENaC blockage. This image was created using BioRender.
Figure 4

A. pH$_U$ over time with arrows indicating changes.

B. [K$^+$] over time with arrows indicating changes.

C. Bar graph showing mean pH$_U$ with asterisks indicating significance:
   - WT
   - dKO
   - dKO control

D. Mean pH$_U$ changes with asterisks indicating significance:
   - WT
   - dKO
   - dKO control

E. NH$_4^+$ + TA excretion (μmol h$^{-1}$) with asterisks indicating significance:
   - WT
   - dKO
   - dKO control

F. ΔNH$_4^+$ + TA excretion (μmol h$^{-1}$) with asterisks indicating significance:
   - WT
   - dKO
   - dKO control
Figure 5

A. K⁺ excretion (µmol/h)

B. ΔK⁺ excretion (µmol/h)

C. Na⁺ excretion (µmol/h)

D. ΔNa⁺ excretion (µmol/h)

E. Urine flow (µl/g BW/5 min)

F. ΔUrine flow (µl/g BW/5 min)
Figure 6

A

$\alpha$-ENaC

\[\text{dKO mice} \quad \text{WT mice}\]

\[\text{90 kDa} \quad \text{90 kDa}\]

\[\text{~34 kDa} \quad \text{~34 kDa}\]

\[\text{\textit{\textcopyright}EnaC 90 kDa} \quad \text{\textcopyrightEnaC 34 kDa}\]

\[\text{\textit{\textcopyright}EnaC 90 kDa} \quad \text{\textcopyrightEnaC 90 kDa}\]

B

$\gamma$-ENaC

\[\text{dKO mice} \quad \text{WT mice}\]

\[\text{95 kDa} \quad \text{70 kDa}\]

\[\text{\textit{\textcopyright}EnaC 95 kDa} \quad \text{\textcopyrightEnaC 70 kDa}\]

\[\text{\textit{\textcopyright}EnaC 95 kDa} \quad \text{\textcopyrightEnaC 70 kDa}\]
Figure 7

A. pH 6

B. pH 7

C. pH 8

D. IC₅₀ (µM benzamil)

E. Residual ATPase activity (%)

(Pig gastric α1 H⁺, K⁺-ATPase)

(Pig kidney Na⁺, K⁺-ATPase)

(Pig gastric α1 H⁺, K⁺-ATPase)

(Pig gastric α1 H⁺, K⁺-ATPase)

(Pig kidney Na⁺, K⁺-ATPase)
Figure 8

Normal

Low K⁺ diet

Low K⁺ diet + benzamil

Na⁺

ENaC

ROMK

K⁺

H⁺

H⁺,K⁺-ATPase

α-IC

pHurine

ENaC

ROMK

K⁺

H⁺

H⁺,K⁺-ATPase

α-IC

pHurine

Benzamil

ENaC

ROMK

K⁺

H⁺

H⁺,K⁺-ATPase

α-IC

pHurine
Benzamil-mediated urine alkalization is caused by the inhibition of H\(^+\), K\(^+\) ATPases

**METHODS**

We studied, if the marked benzamil-induced urine alkalization is mediated by an acute reduction in H\(^+\) secretion via the renal H\(^+\), K\(^+\)-ATPases (HKA). The experiments were performed *in vivo* on double knock-out mice (lacking both H\(^+\), K\(^+\)-ATPase isoforms) and wild type mice. Alterations in dietary K\(^+\) intake were used to change renal baseline H\(^+\), K\(^+\)-ATPase and ENaC activity. The acute effects of benzamil (0.2µg/g BW, sufficient to block ENaC) on urine flow rate, urinary electrolyte and acid excretion were monitored in anesthetized and bladder-catheterized animals.

**OUTCOME:** The 3 key effects of benzamil

CONCLUSION We propose that the benzamil-induced urine alkalization is triggered by a direct inhibition of the HKAs in the collecting duct.