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**Graphical abstract**

![Graphical abstract](http://jci.me/126910/pdf)
Potassium acts through mTOR to regulate its own secretion

Brief title: K+ regulates mTOR

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Conflict of Interest Statement

The authors have declared that no conflict of interest exists.
Abstract

Potassium (K⁺) secretion by kidney tubule cells is central to electrolyte homeostasis in mammals. In the K⁺ secretory “principal” cells of the distal nephron, electrogenic Na⁺ transport by the epithelial sodium channel (ENaC) generates the electrical driving force for K⁺ transport across the apical membrane. Regulation of this process is attributable in part to aldosterone, which stimulates the gene transcription of the ENaC-regulatory kinase, SGK1. However, a wide range of evidence supports the conclusion that an unidentified aldosterone-independent pathway exists. We show here that in principal cells, K⁺ itself acts through the type 2 mTOR complex (mTORC2) to activate SGK1, which stimulates ENaC to enhance K⁺ excretion. The effect depends on changes in K⁺ concentration on the blood side of the cells, and requires basolateral membrane K⁺-channel activity. However, it does not depend on changes in aldosterone, or on enhanced distal delivery of Na⁺ from upstream nephron segments. These data strongly support the idea that K⁺ is sensed directly by principal cells to stimulate its own secretion by activating the mTORC2-SGK1 signaling module, and stimulate ENaC. We propose that this local effect acts in concert with aldosterone and increased Na⁺ delivery from upstream nephron segments to sustain K⁺ homeostasis.
**Introduction**

In mammals, maintaining extracellular potassium ion (K\(^+\)) concentration, [K\(^+\)], within a narrow range is essential for normal cell functions and the ability to sustain life (1). The problem of tight [K\(^+\)] regulation is compounded by the fact that extracellular K\(^+\) is a small fraction of total body K\(^+\), and hence [K\(^+\)] could in principle be subject to large fluctuations, particularly in the face of a sudden increase in intake. Thus, mammals have developed efficient mechanisms to respond to changes in extracellular [K\(^+\)], substantially through rapid and profound changes in K\(^+\) excretion by the kidneys. A combination of systemic (predominantly hormonal) and local renal signaling mechanisms have evolved to rapidly alter K\(^+\) secretion into the kidney tubule lumens by principal cells (PCs) of the distal segment frequently referred to as the aldosterone-sensitive distal nephron (ASDN). The apical translocation of K\(^+\) into the tubule lumen of PCs is mediated predominantly by the renal outer medullary K\(^+\) channel (ROMK). The epithelial Na\(^+\) channel (ENaC), which depolarizes the apical membrane, is central to establishing the driving force for K\(^+\) movement (1, 2). Hormonal regulation of this process is predominantly by aldosterone, a steroid hormone, which acts through the mineralocorticoid receptor (MR) to regulate the transcription of multiple genes that control ENaC activity (3).

A key target of MR gene regulation is the serine-threonine kinase, SGK1, which stimulates apical localization of ENaC and enhances electrogenic Na\(^+\) reabsorption (4). The absence or inhibition of SGK1 activity results in Na\(^+\) wasting and hyperkalemia in mice, despite markedly elevated aldosterone, which recapitulates many of the features of MR deficiency (5). To be activated, SGK1 must undergo phosphorylation within a short hydrophobic motif (HM) near its C-terminus, which is mediated by the kinase mTOR, specifically in its type 2 multi-protein complex, mTORC2 (6). The importance of mTORC2-dependent SGK1 phosphorylation in
regulating ENaC activity and K⁺ excretion is well established (7-9), however, the physiologically relevant control mechanisms have remained poorly characterized.

Additional genetic and physiological evidence also supports the idea that K⁺ acts through non-aldosterone-dependent mechanisms to regulate K⁺ excretion. Notably, even in the complete absence of aldosterone in mice (due to aldosterone synthase gene deletion), the K⁺ excretory response to moderate changes in K⁺ intake is near-normal (10). Similarly, dogs subjected to adrenalectomy and maintained on a low fixed level of aldosterone respond effectively to KCl infusion with increased K⁺ excretion, which is further stimulated by exogenous aldosterone (11).

One mechanism that contributes to this aldosterone-independent regulation is the local effect of [K⁺] to directly inhibit reabsorption of Na⁺ and Cl⁻ in the distal convoluted tubule of the kidney (12-14), a tubule segment just upstream of the ENaC-expressing ASDN. Na⁺ and Cl⁻ are reabsorbed together in an electroneutral fashion in this nephron segment by the sodium chloride cotransporter (NCC) (15). Its inhibition allows more Na⁺ to pass to tubular segments further downstream where ENaC-mediated Na⁺ reabsorption establishes the lumen negative potential driving K⁺ secretion (12, 16, 17). However, pharmacologic inhibition of NCC is insufficient to enhance K⁺ excretion, despite a marked increase in Na⁺ delivery to the ASDN (18, 19); this stands in marked contrast to a KCl load, which rapidly stimulates both a natriuresis and marked kaliuresis (12, 20).

Taken together, these observations suggested the hypothesis that an acute K⁺ load acts to stimulate ENaC activity in PCs, and that this effect precedes and then is augmented by the ENaC-stimulatory effects of aldosterone. We further hypothesized that the mTORC2-SGK1 signaling module mediates these direct K⁺ effects, and integrates them with those of aldosterone. Our present data, based on in vivo balance studies, kidney tubule patch clamp experiments, and cell culture ion
transport and kinase signaling studies support the idea that K\(^+\) acts through basolateral membrane K\(^+\) channels of PCs to stimulate apical membrane ENaC activity by enhancing mTORC2-mediated phosphorylation of SGK1. Our data further implicate WNK1 as an essential component of this signaling pathway, interestingly, independently of its kinase activity. We propose a model in which a WNK1-mTORC2-SGK1 signaling module integrates the effects of aldosterone with local effects of K\(^+\) to regulate K\(^+\) secretion and maintain K\(^+\) homeostasis.
Results

*KCl gavage or intravenous infusion acutely stimulates ENaC-dependent Na⁺ transport in mice.*

In order to assess the role of ENaC activity in the regulation of K⁺ secretion in mice, we examined the effect of K⁺ on ENaC-mediated Na⁺ reabsorption, using the highly specific ENaC inhibitor, benzamil. The difference between Na⁺ excretion in the presence and absence of intraperitoneal (IP) benzamil injection (benzamil-induced natriuresis (BIN)) has been widely used as a reflection of the amount of Na⁺ that is being reabsorbed via ENaC (20, 21). K⁺, administered by gavage, stimulates BIN in both male NMRI and female (C57Bl/6) mice (data summarized in table 1). In the male NMRI mice receiving a K⁺-free gavage, pretreatment with benzamil induced a natriuresis of 1.8±0.4 µmol Na⁺/g BW over 4 h (Figure 1A, difference between vehicle and benzamil curves). The BIN was approximately 3-fold greater (5.7±0.9 µmol Na⁺/g BW over 4 h) in mice receiving KCl gavage (10 µmol K⁺/g BW) (Figure 1B). The effect in spot urine collection was significant as early as 30-60 minutes post K⁺ load (Figure 1B). A similar effect of KCl was observed in female C57Bl/6 mice (Table 1). That this stimulatory effect of KCl on ENaC activity is linked to increased K⁺ excretion can be seen as benzamil suppressible kaliuresis. Consistent with earlier reports (22), K⁺ excretion was stimulated 5-fold by KCl gavage (from 1.8±0.4 to 9.5±1.2), and this effect was markedly reduced by benzamil (Figure 1, C and D and Table 1). During this early phase response, we did not detect a change in total ENaC subunit protein or α- and γ ENaC cleavage products in the kidneys harvested 1 hour post gavage (Supplemental Figure 1).

To investigate if the effect of KCl load relies on signals originating from the GI tract, and to better assess the kinetics, we infused KCl directly into tail veins of anesthetized mice with urine collected via bladder catheter. An acute shift of infusion from NaCl to KCl resulted in a significant 2-fold increase in BIN within 25 minutes, while Na⁺ excretion in the vehicle treated mice was not
altered (compare benzamil and vehicle treated groups in Figure 1, E and F). As with the gavaged mice, intravenous infusion of KCl also induced a kaliuresis, which was markedly suppressible by benzamil (Figure 1, G and H).

*KCl-mediated increase of benzamil-induced natriuresis is not a product of augmented Na\(^+\) delivery to ENaC.*

The above data support the conclusion that KCl gavage or infusion stimulates ENaC as reflected by increased BIN. Furthermore, this activation of ENaC is essential for enhancing K\(^+\) excretion as reflected by the marked reduction in K\(^+\) excretion in benzamil-treated mice (Figure 1D). Previous work supports the conclusion that selectively increasing Na\(^+\) delivery by inhibiting NCC with thiazides is not sufficient to enhance ENaC dependent K\(^+\) excretion (18), consistent with the idea that activation of ENaC per se is necessary. In order to further investigate the potential role of increased Na\(^+\) delivery to the channel, we administered NaCl by gavage to induce a natriuresis without inducing hypovolemia. Under these conditions, NaCl induced a marked natriuresis (significantly greater than that induced by KCl) (Supplemental Figure 2), however, in sharp contrast to KCl (Figure 1, A and B), it did not stimulate BIN (Figure 2, A and B). Thus, increased delivery of Na\(^+\) to ENaC does not by itself alter benzamil inducible natriuresis.

*Acute KCl stimulation of ENaC is mineralocorticoid receptor (MR) independent.*

Together with prior literature, the above findings support the conclusion that K\(^+\)-induced K\(^+\) excretion requires ENaC stimulation. As noted above, it is well established that K\(^+\) stimulates aldosterone secretion by the adrenal cortex, and that aldosterone acts in turn through the mineralocorticoid receptor (MR) to stimulate ENaC (3). This effect is clearly important for long-
term responses to large potassium loads, however, it may not be necessary for the early response to more physiological K\(^{+}\) loads (10, 11). In support of the idea that the rapid activation of ENaC by KCl (Figures 1, B and F) was not elicited by aldosterone, we found that plasma aldosterone level in mice receiving control vs. KCl were not significantly different 1 h post gavage (Table 1). We then went on to directly assess the role of MR using the MR antagonist, eplerenone. As shown in Figure 2, C and D, we found that KCl still stimulated BIN in mice pretreated with eplerenone (compare Figure 2, C and D). Indeed, the effect of KCl in mice treated with eplerenone was not significantly different from its effect in vehicle-treated controls (compare Figures 1, B and D).

**Effect of extracellular [K\(^{+}\)] on ENaC activity in native mouse CCD and cultured CCD cells.**

The above data strongly support the idea that K\(^{+}\) can act independently of changes in aldosterone, MR activation, or signaling from the GI tract to rapidly modulate ENaC and regulate its own excretion. In order to begin to address the underlying mechanism, we next performed in vitro patch clamp experiments on freshly isolated cortical collecting duct (CCD). Mouse CCD was dissected in normal K\(^{+}\) buffer (5 mM), as described (23), tubules were split open, and whole cell patch conditions were established. After achieving a stable current at -60 mV, extracellular [K\(^{+}\)] was reduced to 1 mM by replacing 4 mM of KCl with NaCl, with all other buffer conditions unchanged. As shown in Figure 3A, reducing bath [K\(^{+}\)] to 1 mM resulted in a significant decrease in amiloride sensitive current measured after ~15 minutes, consistent with the in vivo results.

We next examined the effect of changing [K\(^{+}\)] in mpkCCD cells, a stable cell line that reproduces all of the key features of native CCD (24). In initial experiments, in order to achieve maximal distinction between low and high [K\(^{+}\)], we adapted the cells to DMEM containing 1 mM KCl and 144 mM NaCl without serum for 2 h. At t = 0, the medium was changed to the K\(^{+}\)
concentrations shown (Figure 3B), with inverse change of \([\text{Na}^+]\) to maintain constant osmolarity and total cation concentration. Consistent with both the in vivo balance studies and patch clamp experiments, amiloride-sensitive current increased by \(47 \pm 0.27\%\) when \([\text{K}^+]\) was increased to 3 mM and by \(88 \pm 1.58\%\) when increased to 5 mM. Interestingly, there was no further increase in current when \([\text{K}^+]\) was increased to 6, 7 or 10 mM. Similar results were obtained when \(\text{K}^+\) was replaced by choline chloride rather than \(\text{NaCl}\) (data not shown). An inverse effect was found when cells were adapted to 5 mM \(\text{K}^+\) and shifted to 1 mM (data not shown), consistent with the patch clamp results in intact tubule.

In order to examine more physiologically relevant \(\text{K}^+\) concentrations, comparable to those achieved in the in vivo experiments, we adapted the mpkCCD cells to 3 mM \(\text{K}^+\), and the added KCl to bring the \([\text{K}^+]\) up to 6 mM. As shown in Figure 3C, a significant increase in ENaC current was seen. The effects were less dramatic than those seen with larger changes in \([\text{K}^+]\), but were consistent and highly statistically significant. Together, these data strongly support a direct local effect of \(\text{K}^+\) to stimulate ENaC by increasing SGK1 activity in CCD cells.

**SGK1 activity is required for \(\text{K}^+\) stimulation of ENaC.**

Previous literature has established that SGK1 is an important regulator of ENaC, and mediator of effects of aldosterone to stimulate \(\text{K}^+\) excretion (25). SGK1 stimulates ENaC in a variety of experimental systems (25), and SGK1 KO mice fail to appropriately activate ENaC in response to increased dietary \(\text{K}^+\), and become hyperkalemic despite marked elevation of aldosterone (26). It is well established both in vitro and in vivo that mTOR is required for ENaC activity, and that its effects proceed at least in part through SGK1, which it phosphorylates within its C-terminal HM (7). Some activators of mTOR-dependent HM phosphorylation such as insulin and growth factors
have been identified (4, 7), however, the role of regulated HM phosphorylation in physiological feedback mechanisms, particularly those that control extracellular [K+] remain poorly characterized. With these considerations in mind, we first examined whether changing [K+] altered SGK1 HM phosphorylation. Immunoblots from the mpkCCD cells in Figure 3, B and C were prepared and stained with anti-phospho-SGK1/S422. As shown in Figure 4, A and B, SGK1 S422 phosphorylation increased proportionately to the increase in amiloride-sensitive current (Figure 3, B and C, respectively) consistent with a role for SGK1 HM phosphorylation in ENaC stimulation by K+.

A role for SGK1 to stimulate ENaC in response to local changes in extracellular [K+] was further supported by the effects of SGK1 inhibition with GSK650394 (27), which blocked the increase in current induced by shifting media [K+] from 1 mM to 5 or 8 mM (Figure 4C) and from 3 mM to 6 mM (Figure 4D).

*mTORC2 activity is required for K+-stimulated SGK1 phosphorylation and activation of ENaC in native CCD and cultured cells.*

We next asked if mTOR-dependent phosphorylation of SGK1 is required for K+-stimulated activation of ENaC by performing a series of inhibitor and knockout experiments in native tubules and cultured cells. We first performed patch clamp experiments in native CCD treated with AZD8055, a potent and specific inhibitor of mTOR in both of its major complexes, mTORC1 and mTORC2. As shown in Figure 5A and Supplemental Table 1, AZD8055 suppressed current, as previously described (8), and abolished the difference in amiloride-sensitive current seen in 1 vs. 5 mM K+. Similarly, in mpkCCD cells, AZD8055 blocked the ENaC-stimulatory effect of increasing [K+] from 1 to 5 mM (Figure 5B), in parallel with blocking SGK1 S422 phosphorylation.
mTORC1 and mTORC2 both contain mTOR, but due to the specificity conferred by associated proteins such as Raptor (mTORC1), Rictor (mTORC2) and mSin1 (mTORC2), they respond to distinct stimuli and phosphorylate distinct targets (28). The bulk of data support the idea that under most conditions SGK1 is phosphorylated by mTORC2 (6, 7, 29). However, there are data suggesting that in some cases SGK1 is phosphorylated by mTORC1 (30, 31). To address this issue in the context of K⁺ regulation, we treated the mpkCCD cells with the mTORC1-specific inhibitor rapamycin, which had no effect on K⁺-induced current or SGK1 phosphorylation (data not shown). This result is consistent with the idea that the K⁺ effect is mediated by mTORC2, however, the possibility remains that mTORC1 and mTORC2 are redundant in this regard, and both must be inhibited (as by AZD8055). In the absence of a specific pharmacologic inhibitor of mTORC2, we used the CRISPR-Cas9 system to knockout the mTORC2-specific component, mSin1 in HEK-293T cells, as described (32). We found that wild type HEK-293T cells (SIN1⁺/⁺) respond to an increase in extracellular [K⁺] with a robust increase in SGK1 phosphorylation (Supplemental Figure 3), which was lost in the mSin1 KO (HEK-293T SIN1⁻/⁻) cells. Notably, phosphorylation of the mTORC1-specific target, p70-S6-kinase was unaffected (data not shown and (32)). Transient transfection of mSin1 restored the effect of K⁺. In contrast, despite being mTORC2-dependent, phosphorylation of Akt (a close relative of SGK1) at S473 was unaffected by changes in [K⁺] (Supplemental Figure 3). Together with the native tubule and mpkCCD data, these data strongly support the idea that K⁺ acts locally within PCs to stimulate mTORC2-dependent SGK1 phosphorylation and thereby increase ENaC activity. Interestingly, this effect does not reflect generalized activation of mTORC2, but rather selective activity toward SGK1.
Effects of [K+] on ENaC are mediated by basolateral K+ channels, most likely Kir4.1/5.1.

The basolateral—but not the apical—membranes of PCs are exposed to K+ concentrations that reflect plasma levels. Thus, a physiologically relevant effect on ENaC should be triggered by changing [K+] on the basolateral but not the apical side of the cells. We tested this by selectively altering basolateral vs. apical [K+] in Transwells. As shown in Figure 6, in mpkCCD cells, ENaC current (Figure 6A) and SGK1 phosphorylation (Figure 6B) were stimulated by increasing basolateral but not apical [K+], while an inverse effect was elicited by selectively decreasing basolateral [K+] (Supplemental Figure 4).

The resting potential of PC basolateral membranes is dominated by K+ channels, predominantly, Kir4.1/5.1 (33). In order to determine if basolateral K+ channels were implicated in the effects of [K+], we treated mpkCCD cells adapted to 1 mM [K+] with BaCl2 on the basolateral side, just prior to changing [K+], which was either raised to 5 mM or kept at 1 mM (Figure 7, A and B). In cells maintained in 1 mM K+, addition of Ba2+ stimulated amiloride-sensitive current (Figure 7A) and SGK1 phosphorylation (Figure 7B) comparably to raising [K+] to 5 mM. Ba2+ did not provide additional stimulation beyond that of 5 mM [K+], further supporting the conclusion that the effects of this signaling system plateau at K+ concentrations in the high physiologic range.

We further addressed the specific K+ channel(s) by treating the cells with a newly developed inhibitor specific for Kir4.1, VU0134992 (34) (Figure 7, C and D). The effects found were similar to those of Ba++, providing strong support for the idea that Kir4.1 (probably in the form of the heteromultimer Kir4.1/5.1) is part of a signaling system that mediates direct sensing of [K+] by the ASDN.

Potential role of WNK1 in linking changes of basolateral [K+] to SGK1 activation.
The foregoing data support a mechanistic picture in which changes in basolateral [K+] alter the K+ reversal potential, which results in altered membrane potential as K+ moves through Kir4.1/5.1 channels. This somehow results in altered mTORC2-dependent phosphorylation of SGK1, with concomitant change in ENaC activation by Nedd4-2. However, how a change in membrane potential alters mTORC2-dependent SGK1 phosphorylation is still unclear. In this context, the recently described effect of [K+] to alter WNK kinase activities in the DCT might be relevant. WNKs are well known for their central role in a kinase cascade which proceeds through phosphorylation of the kinase SPAK, which in turn phosphorylates and activates NCC (35). Low [K+] stimulates—and high K+ inhibits—this activity, at least in part by altering the intracellular concentration of Cl− (14). WNK1 and WNK4 both bind Cl−, which inhibits their kinase activities (36). Other studies have suggested a role for WNKs in regulating K+ secretion within the ASDN, which expresses high levels of WNK1, and moderate levels of WNK4 (37, 38).

As a first step toward assessing a potential role of WNKs in mediating the effects of [K+] on SGK1, we examined SGK1 phosphorylation in WNK1-deficient HEK-293T cells (HEK-293T WNK1−/−) a kind gift of Dr. Arohan Subramanya (39). K+-stimulated SGK1 phosphorylation was nearly undetectable in these cells, and was fully rescued by heterologous WT or kinase dead (K233M) WNK1 (1-491) (Figure 8, A and B). In contrast, Akt phosphorylation (S473) (also mTORC2-dependent; Figure 8A and (40)) was unaffected by elimination of WNK1. Thus, the effect of WNK1 is not a generalized effect on mTORC2, but rather selectively modulates its activity toward SGK1. These findings are consistent with a role of WNK1 acting through a non-catalytic mechanism to modulate K+-regulated mTORC2-dependent SGK1 phosphorylation, as shown schematically in Figure 9.
Discussion

It is well established that mammals respond to an acute K+ load with a profound and rapid increase in secretory K+ excretion, which is mediated almost entirely by the distal segments of the renal tubules, and occurs through apical membrane K+ channels (1). The driving force for apical K+ movement is established by electrogenic Na+ transport through ENaC (22). The steroid hormone aldosterone is essential for this process to proceed normally, particularly with chronic K+ loading or when large quantities of K+ must be excreted (10, 41). However, increasing evidence supports the idea that regulated K+ secretion can occur to a significant extent independently of changes in aldosterone, particularly in response to the kind of K+ load seen with a naturally occurring K+ rich meal (10, 11, 42). Recent data support the idea that direct inhibition by K+ of electroneutral Na+-Cl- cotransport via NCC contributes to K+ secretion by enhancing Na+ delivery to the ENaC-expressing, K+ secretory segments (12, 14, 16, 43). However, NCC inhibition by itself is not sufficient for this rapid effect (18). Together, these observations suggest the hypothesis that ENaC might be acutely activated by local changes in [K+]p.

Our present data strongly support the idea that K+ is sensed and acts locally within PCs, independently of changes in aldosterone, to rapidly stimulate ENaC and thereby enhance its own secretion. First, we find in mice that a K+ load begins to enhance benzamil-inducible Na+ excretion (a reflection of ENaC activity) prior to significant change in aldosterone level, and that a substantial proportion of this early effect remains intact in the face of pharmacologic blockade of MR. This increase in ENaC activity also is not due to increased Na+ delivery to the channel in that it is not duplicated by increasing Na+ delivery through increased Na+ load. Second, in patch clamp experiments on isolated CCDs, selectively lowering [K+]p inhibits ENaC activity. Third, in cultured CCD cells, raising basolateral [K+]p in the culture medium rapidly stimulates—and lowering [K+]
inhibits—amiloride-sensitive Na\(^+\) current. That these acute effects on ENaC are implicated in controlling K\(^+\) excretion in vivo is supported by the observation that benzamil inhibits K\(^+\) excretion with a time course and extent that parallel its stimulation of Na\(^+\) excretion (compare Figure 1, B and D), similar to prior reports (22).

As a first step toward characterizing the mechanism underlying this local regulation, we have shown in cultured CCD cells grown on Transwell filters, that the effect is seen only with manipulation of basolateral (but not luminal) [K\(^+\)]. We further observed that inhibition of basolateral K\(^+\)-channels with either the non-specific K\(^+\) channel inhibitor, Ba\(^{2+}\), or with a specific inhibitor of Kir4.1 (VU0134992), mimics the effect of high [K\(^+\)], supporting the idea that Kir4.1-dependent basolateral membrane potential is integral to the underlying mechanism.

Our data further establish the central importance of the mTORC2-SGK1 signaling module in mediating these effects. SGK1 is a well-established ENaC regulator, which is under dual regulation: Its gene expression is rapidly and potently stimulated by aldosterone, the biosynthesis of which is tightly regulated by [K\(^+\)]. Aldosterone acts through MR to stimulate SGK1 gene transcription, and ultimately control its level of protein expression. However, SGK1 kinase activity requires mTORC2-dependent phosphorylation at S422 (within its C-terminal HM) (6), and acute inhibition of mTOR inhibits ENaC in both cultured cells (7) and in vivo (8). ENaC is stimulated substantially through the effect of SGK1 to phosphorylate and thus inhibit the ubiquitin ligase, Nedd4-2 (44), although other effects of SGK1 to regulate ENaC may contribute, for example direct phosphorylation of the channel (45). We find that K\(^+\) stimulates mTORC2-dependent SGK1 phosphorylation, and that this effect is required for activation of ENaC. In addition to ENaC, it is also important to note that a role for regulation of K\(^+\)-channels themselves, in particular ROMK, cannot be ruled out (46). Indeed, one recent report found that genetic disruption of mTORC2 (via
constitutive Rictor knockout) caused hyperkalemia by disrupting both ENaC and ROMK (9). Although this may play a role in chronic regulation, the bulk of evidence supports the idea that acute regulation proceeds predominantly through ENaC-dependent control of the electrical gradient that drives K⁺ secretion (22).

It is interesting to note the similarities and differences between our present findings and those of other groups focusing on K⁺ effects in the DCT (47-49). In both cases, changing [K⁺] has cell-autonomous effects on apical transporters, ENaC on the one hand, NCC on the other. In both cases, the effect appears to be induced by basolateral but not apical changes in [K⁺], and to depend on basolateral K-channels (possibly Kir4.1/5.1) (50). However, in the two cases, the effects of K⁺ are opposite: NCC is inhibited by high [K⁺], ENaC is stimulated. Both of these effects are physiologically rational, however, the mechanistic bases must diverge. WNK proteins play a well-established role to mediate K⁺-induced inhibition of NCC, while our present data suggest that WNK1 (and potentially WNK4) plays a key role in the effects of K⁺ to selectively stimulate mTORC2 phosphorylation of SGK1 (Figure 8). In striking contrast to K⁺ regulation of SPAK and NCC, the K⁺ effect on SGK1 phosphorylation does not require WNK1 kinase activity (Figure 8). Furthermore, the effect appears to be specific for SGK1, and not a generalized activation of mTORC2 since SGK1’s close relative, Akt2 (also a substrate for mTORC2), is unaffected (Figure 8A and (40)). This latter observation is reminiscent of our recent findings that angiotensin II selectively stimulates SGK1 but not Akt phosphorylation (32). Of further interest, we found that changes in subcellular localization were central to this selective regulation: hormone-induced movement of mTORC2 and SGK1 from a perinuclear to plasma membrane location was required for selective regulation. With this in mind, it is notable that recent work from Boyd-Shiwarski et al. (51) demonstrated similar changes in WNK bodies in response to K⁺, in both DCT and CNT.
cells. It will be of interest to explore these relationships in future work. Taken together with prior work from Cobb and colleagues (37), our present findings suggest that WNK1 serves as a K+-regulated scaffold that promotes mTORC2-dependent phosphorylation of SGK1 (Figure 9). Although it is appealing to speculate that Cl⁻ binding to WNK1 plays a role as it does in NCC regulation (14, 36), our current data are ambiguous. In any case, our present data suggest a parsimonious explanation for shifting Na⁺ transport from electroneutral to electrogenic, particularly in DCT2 cells, which express both NCC and ENaC. Further experiments are needed to explore the role of Cl⁻ and to characterize the precise mechanism of this WNK-mTORC2-SGK1 signaling module.
Methods

(please see Supplemental Information for additional details)

**Mice.**

*In vivo experiments performed in un-anesthetized animals:* We used male (NMRI) and as a control for gender or strain specific effects female (C57Bl/6) mice. All mice were purchased from Janvier Labs, Le Genest-Saint-Isle, France. Mice were transported to Aarhus in a temperature controlled (21 °C) car. Animals were acclimatized to the housing facility in Aarhus for at least 1 week before used in experiments. The mice were fed a control diet (ssniff®EF R/M Control diet (0.9 % K+ and 0.2 % Na+), ssniff Spezialdiäten, Germany.

*Infusion and native CCD patch clamp experiments:* Male C57BL/6 mice were used. The mice were fed a low sodium diet for 10 days (TD90228, 0.01-0.02% NaCl) prior to experiment. All mice experienced a 12/12-hour light/dark cycle and had access to normal drinking water and a control diet ad libitum. All mice were 8-12 weeks of age at the time of the experiments. In order to prevent circadian aldosterone fluctuations from affecting our data all animal experiments were started at 9 am.

**Electrolyte gavage.**

Mice were either given a K+, Na+ or control gavage. To initiate a prandial metabolic response, i.e. rise in insulin all gavages contained 2% mass glucose to. The K+ gavage contained 2% mass K+ (512 mM) and Cl-. This maneuver is well described to increase plasma K+, reduce NCC phosphorylation and induce natriuresis and kaliuresis acutely (12, 20). The Na+ gavage had the same osmolarity (512 mM) and Cl- concentration as the K+ gavage. The control gavage only
contained glucose. The mice received a volume of 20 µL/g and a total K⁺ load that corresponds to approximately 25% of daily K⁺ intake (52). Gavage was always given at 9 AM and urine was collected as spot urine during the following 4 hours. During the 4-hour gavage experiments the mice only had access to water.

**Benzamil injections:** Benzamil is a specific ENaC antagonist (53). 0.2 µg/g BW benzamil (0.4mg/ml dissolved in 0.9 % saline with 0.5 % DMSO) or vehicle (5 % DMSO in 0.9 % saline) was administrated via intraperitoneal (i.p.) injections 15 minutes prior to gavage. This dose has been validated to inhibit ENaC efficiently and specific (18)

**Spot urine in metabolic cages.**

Before gavage, the mice were weighed to adjust electrolyte and benzamil dose. Post gavage animals were placed in metabolic balance cages. Urine samples collected during handling of the mice prior to gavage were used as baseline values. Urine samples were collected as spot urine, as previously described (20).

**KCl infusion.**

Mice were given a single water load (25 µl/g BW) as gastric gavage and then anesthetized with a i.p. injection of ketamine and xylazin. Anesthesia was maintained by repetitive bolus injection with a third of the induction dose every 30 min. While the mice were placed on a thermostatic controlled heating plate (38°C) an intravenous catheter was inserted in a tail vein and continuous infusion of 300 mOsm saline was started. The lower part of the abdomen was then incised, and the urinary bladder was identified and catheterized. Urine was collected every 5 minutes for flame photometry. The mice were randomly divided in two groups. In one group the saline infusion was
switched to a 300 mOsm KCl after the first 30 minutes at a rate of 4 μl/g BW/h. The infusion rate was titrated to reach [K+]₀ of ~6 mM. In the second group the saline infusion was continued throughout the experiment. Simultaneously with changes of infusate, all animals received an i.p. bolus injection of benzamil or vehicle.

MR blockade.

Eplerenone is a specific MR antagonist with relative long half-life in in vivo use (54). Eplerenone tablets (Teva pharma, Sweden) were dissolved in tap water (20 mg eplerenone/ml) and administrated by gavage. Mice were given 200 μg/g BW daily (divided into two doses of 100 μg/g BW at 9:00 am and 9:00 pm) for 4 days prior to experiments and 100 μg/g BW at 9:00 on the day of the experiments. Efficacy of the MR blockade using this protocol has previously been verified in our laboratory (52).

Flame photometry.

The concentrations of Na⁺ and K⁺ in the urine samples were measured using a flame photometer (Model 420 Flame Photometer, Sherwood Scientific, Cambridge, UK) on a 3 mM LiCl background. In order for the samples to be in the detection range, the samples were diluted 1:4 in ddH₂O.

Plasma electrolyte and aldosterone measurements and tissue harvest.

45 minutes post gavage, mice were anesthetized using ketamine and xylazin i.p. injections. One hour post gavage blood was sampled by retroorbital puncture using a heparinized capillary tube. Blood was analyzed immediately in an blood gas and osmolyte analyzer. Subsequently whole
blood was extracted from the right atrium of the heart by a heparinized syringe and centrifuged at 4600 g for 2 minutes. The plasma was kept at -80°C plasma aldosterone was measured using an ELISA kit.

After blood collection, mice were perfused with 20 ml PBS via the left atrium of the heart. The kidneys were then removed, decapsulated and then snap-frozen in liquid nitrogen and kept at -80°C until use.

*Patch-clamp and whole cell current measurement in native CCD.*

The collecting tubules were dissected from the kidney slices, split open and prepared for apical membrane patch clamp, as previously described (55). An Axon 200A amplifier was used to measure the whole-cell Na⁺ currents in CCD. After forming a gigaohm seal, the bath solution was changed to either 1 mM or 5 mM external K. Measurements were made after currents reached steady state (15 min after change of bath). ENaC current was assessed as amiloride-inhibitable current at clamp potential of -60 mV. Data were analyzed using the pClamp software system 9.0 (Axon). For inhibitor study experiments, AZD8055 (1 μM) or equal volume of vehicle was added in the bath solution, following which bath [K⁺] was changed to either 1 mM or 5 mM in presence of AZD8055 or vehicle. Once stable current was reached, whole cell measurements were made at a holding potential of -60 mV.

*Cell culture, transfection and Treatment.*

mpkCCDc14 (mpkCCD) cells were maintained in modified DMEM/Ham’s F12 (1:1) medium (“regular medium”) as described previously (56).
HEK-293T, HEK-293T WNK1+/− (39) and HEK-293T SIN1−/− (32) cells were grown in DMEM (DME H-21, CCFAA005, UCSF Cell culture facility) supplemented with 10% FBS and 1% penicillin, and streptomycin antibiotics. Cells were transfected using Polyethylenimine (PEI). For experiments with different K⁺ concentrations, DMEM without KCl was used. K⁺-free DMEM was supplemented with KCl, NaCl or Choline Chloride to get the desired concentrations of K⁺ in the media as indicated in the figure legends.

Measurement of ENaC-Dependent Na⁺ Transport.

For electrophysiological measurements, mpkCCDc14 cells were seeded on type I collagen-coated filters (Transwell, pore size 0.4 μm, Corning Costar) until the cell monolayers reached transepithelial resistance >1,000 Ω.cm². They were then maintained in serum-free plain DMEM for at least 16 h before treatment with aldosterone (1 μM for 3 h) to induce endogenous SGK1 expression. For extracellular [K⁺] stimulation/recovery experiments, cells were adapted for at least 2 h to 1 mM or 3 mM [K⁺]. At t=0, the medium [K⁺] was increased by addition of KCl or equimolar Choline Chloride or sodium chloride and incubated for 1 h prior to measurement of amiloride sensitive current. Transepithelial resistance and potential difference across the cell monolayer were measured using Millopep ERS voltohmmeter (MilliCell ERS; Millipore). The equivalent short-circuit current was calculated using Ohm’s law. Amiloride (10 µM) was added to the medium on the apical side at the end of the experiments to derive amiloride-sensitive component of the current. Amiloride almost completely (>98%) inhibited the total current indicating its ENaC dependence. In some experiments, cells were adapted to 5 mM [K⁺] and then transferred to media with different [K⁺] as specified.
In inhibitor study experiments, inhibitors, AZD8055 (1 μM, apical and basolateral sides), GSK650394 (6 μM, apical and basolateral sides), BaCl₂ (100 μM, basolateral side), VU0134992 (10 μM, apical and basolateral sides) or equal volumes of vehicle as the control were added for specified periods of time. Following electrophysiological measurements, cells were harvested and processed for immunoblot analysis.

**Immunoblotting.**

*Cultured cells:* To determine protein expression levels, Western blot analysis was performed as previously described (7). Briefly, cells were lysed using lysis buffer containing complete protease inhibitor cocktail and PhoSTOP phosphatase inhibitor. 40 μg of total protein from each cell extract supernatant was electrophoresed on 7.5% polyacrylamide Gels. The blots were then probed with primary antibodies followed with horseradish peroxidase (HRP)-conjugated secondary antibodies. Each experiment was repeated more than three times. The bands corresponding to pSGK1 and total SGK1 were quantified using Image J software, as previously described.

*Kidney lysates:* Half kidneys were homogenized in 1 ml lysis buffer using a tissuelyser. The supernatant was aspirated and protein concentrations were measured, electrophoresed and blotted as further described in supplemental information.

**Expression Constructs.**

Flag-SGK1, and SIN1-V5, were generated as described previously (57). WT WNK1 (1-491) and WNK1 (1-491) (K233M) were generous gifts from Drs. Melanie H. Cobb and Chou-long Huang (37).
Statistics.

GraphPad Prism (GraphPad Software, USA) and Microsoft Excel were used for data analysis. Comparison between two groups was performed by student’s T-test. Comparison between more than two groups was analyzed by one-way ANOVA with a Bonferroni’s multiple comparison post-test. Comparison of data collected time dependently was done with two-way ANOVA with a Bonferroni’s multiple comparison post-test. Used statistical test are stated in each figure legend. We presuppose normal/Gaussian distribution of the data but did not test for this, because of the low n (6-8) values in our data. Time dependent data are shown as mean ± SEM. Time independent data are shown as bar charts or individual data points in dot plots. Mean ± SEM are depicted as horizontal lines on top of the individual data point. P<0.05 is considered significant and p values are either shown directly in the figures or symbols for p-values are explained in figure legends.

Study Approval.

Animal experiments were performed in accordance with the Danish legislation on the protection of animals (Dyreforsøgstilsynet licence No.:2013-15-2934-00787 and No: 2017-15-0201-001166).
Author contributions

MVS, BS, ISJ, PW, NA, SLS and WHW conducted the experiments and analyzed data. CEG developed SIN1 knockout cell line. CEG and SLS contributed to scientific discussion. MVS, BS and DP designed the study. MVS, BS and DP drafted the manuscript.
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References


Figure 1. Systemic K⁺ loading acutely increases benzamil inducible urinary Na⁺ loss. (A and B) Cumulative Na⁺ excretion relative to BW during 4 hours following control (A) or KCl (B) gavage in vehicle vs. benzamil treated mice. Differences between the curves (marked with black curly bracket) represent benzamil-induced natriuresis (BIN). (C and D) Cumulative K⁺ excretion relative to BW during the first 4 hours post control (C) or KCl (D) gavage in vehicle vs. benzamil treated mice. Differences between the curves (marked with red curly bracket) represent benzamil-suppressible kaliuresis. Dashed line indicates K⁺ load given. (E and F) Cumulative Na⁺ excretion during the first 30 minutes following intravenous saline control (E) or KCl (F) infusion in vehicle vs. benzamil treated mice. Differences between the curves (marked with black curly bracket) represent benzamil-induced natriuresis (BIN). (G and H) Cumulative K⁺ excretion during the first 30 minutes following intravenous saline control (G) or KCl infusion (H) in vehicle vs. benzamil treated mice. Differences between the curves (marked with red curly bracket) represent benzamil-suppressible kaliuresis. In all panels (▲) represents vehicle and (■) represents benzamil treated mice. * indicates p<0.05 between vehicle and benzamil. $ indicates p<0.05 between KCl and control loading by 2-way ANOVA multiple comparison test. N=6 in all presented data.
Figure 2. K⁺ stimulated benzamil-inducible urinary Na⁺ loss is not due to increased Na⁺ delivery to ENaC, and is largely independent of MR signaling. (A and B) Effect of control (A) or NaCl (B) gavage on Na⁺ excretion in vehicle vs. benzamil treated mice. (C and D) Effect of control (C) or KCl (D) gavage on Na⁺ excretion in vehicle vs. benzamil treated mice in the presence of mineralocorticoid receptor blockade by eplerenone. As in Figure 1, differences between curves (marked with black curly brackets) represent benzamil-induced natriuresis (BIN). In all panels (▲) represents vehicle and (■) represents benzamil treated mice. * indicates p<0.05 between vehicle and benzamil. $ indicate p<0.05 between control and KCl gavage by 2-way ANOVA multiple comparison test. N=8 in all presented data.
Figure 3. Extracellular \([K^+]\) regulates amiloride-sensitive \(Na^+\) current. (A) \(K^+\) regulates ENaC activity in native mouse CCD. Mouse collecting tubules were dissected in 5 mM \(K^+\) buffer and subjected to apical membrane patch clamp. The bath \([K^+]\) was changed to either 1 mM or maintained at 5 mM. ENaC current was assessed as amiloride-sensitive current at clamp potential of -60 mV. N=5 mice for each group. *P < 0.05 by two-tailed Student’s T-test. pA, picoamperes. (B and C) \(K^+\) stimulates \(Na^+\) current in mpkCCD cells. Cells were adapted to 1 mM \([K^+]\) (B) or 3 mM \([K^+]\) (C) for 2 h, medium \([K^+]\) was increased by addition of KCl, and incubated for 1 h additional prior to measurement of amiloride sensitive current, as described in Methods. Data are means ± SEM from three independent experiments. *P < 0.05; ****P < 0.0001 by one-way ANOVA (B). **P < 0.01 by two-tailed Student’s T-test (C). μA, microamperes.
**Figure 4. SGK1 activity is required for K⁺ stimulation of ENaC.** (A and B) K⁺ stimulates SGK1 phosphorylation in mpkCCD cells. Cells from Figure 3B and C were lysed and prepared for Western blot, and stained with antibodies as indicated. A and B, upper panels: Western blot images showing blots stained with anti-phospho-SGK1/S422, total SGK1 and α-tubulin as labeled. A and B, lower panels: quantitation of phospho-SGK1/total SGK1 (as described in Methods). Data are means ± SEM from three independent experiments. A, lower panel: *P < 0.05, ***P<0.001 by one-way ANOVA. NS, Not significant. B, lower panel: *P < 0.05 by two-tailed Student’s T-test. (C and D) Inhibition of SGK1 activity decreases K⁺-induced ENaC activity. mpkCCD cells were adapted to 1 mM (C) or 3 mM (D) extracellular [K⁺] as in Figure 3B and C. The SGK1 inhibitor, GSK650394, or vehicle was added and extracellular [K⁺] was then increased by addition of KCl for 1 h prior to determining amiloride-sensitive Na⁺ current. Data are means ± SEM from three independent experiments. ***P<0.001, ****P<0.0001 by one-way ANOVA. μA, microamperes.
Figure 5. mTORC2 activity is required for the effects of extracellular [K⁺] on ENaC.  (A) mTOR inhibition blocks K⁺-induced ENaC current in native CCD. Mouse collecting tubules were dissected in 5 mM [K⁺] buffer and subjected to apical membrane patch clamp, as in Figure 3A. The mTOR inhibitor, AZD8055, was added, following which the bath [K⁺] was changed to either 1 mM or maintained at 5 mM [K⁺]. Whole cell measurements were made at a holding potential of -60 mV. N=5 or 3 mice for each group as indicated in supplemental Table 1. *P<0.05, ** P<0.01, ****P<0.0001 by one-way ANOVA represent the differences are significant between 1 and 5 mM K⁺ and between AZD8055 treatments in comparison to their corresponding controls. pA, picoamperes. (B) mTOR inhibition blocks K⁺-induced ENaC current in mpkCCD cells. Cells were preincubated in 1 mM [K⁺], as in Figure 3B. AZD8055 or vehicle was added as indicated, following which [K⁺] was shifted to 5 mM where shown. Summary graph shown represents effect of AZD8055 on amiloride sensitive Na⁺ current. Data are means ±SEM from three independent experiments. ****P<0.0001 by one-way ANOVA. μA, microamperes. (C) AZD8055 blocks SGK1 S422 phosphorylation in mpkCCD cells. Cells from Figure 5B were lysed and prepared for Western blot, and stained with antibodies as indicated. Left panel: Western blot images showing blots stained with anti-phospho-SGK1/S422, and total SGK1 as labeled. Right panel: quantitation of phospho-SGK1/total SGK1 (as described in Methods). Data are means ±SEM from three independent experiments. **P<0.01, ***P<0.001, ****P<0.0001 by one-way ANOVA.
Figure 6. SGK1 phosphorylation and ENaC current are regulated by basolateral [K⁺]. (A) Effect on amiloride-sensitive current of changing apical (left panel) vs. basolateral (right panel) [K⁺] from 1 mM to 5 mM in mpkCCD cells. Data are presented as means ±SEM. * P<0.05 by two-tailed Student’s T-test. NS, Not significant. (B) Effect on endogenous pSGK1/pS422 level of changing apical vs. basolateral [K⁺] from 1 mM to 5 mM. Upper panel: Western blot images showing blots stained with anti-phospho-SGK1/S422, and total SGK1 as labeled. Lower panels: quantitation of phospho-SGK1/total SGK1 in response to changes in apical (left panel) vs. basolateral (right panel) [K⁺] (as described in Methods). Data are presented as means ±SEM. * P<0.05 by two-tailed Student’s T-test. NS, Not significant. Raising basolateral but not apical [K⁺] stimulates SGK1 S422 phosphorylation and Na⁺ current.
Figure 7. The effect of extracellular \([K^+]\) on SGK1 phosphorylation and ENaC current is mediated by basolateral \(K^+\) channels. (A and B) Effect of \(K^+\)-channel inhibitor, \(Ba^{2+}\), on ENaC current (A) and SGK1 phosphorylation (B). mpkCCD cells were adapted to 1 mM \([K^+]\), and then \([K^+]\) was either raised to 5 mM or kept at 1 mM for 1 h. \(BaCl_2\) (100 µM) or vehicle was added to the basolateral medium 5 min before changing \([K^+]\). Note in particular the effect of \(Ba^{2+}\) to increase \(Na^+\) current and SGK1 phosphorylation in 1 mM \([K^+]\) to levels approaching those seen in 5 mM \([K^+]\). Data are means ±SEM from three independent experiments. **\(P<0.01\), ***\(P<0.001\) by one-way ANOVA. \(\mu A\), microamperes. (C and D) Effect of Kir 4.1 inhibitor, VU0134992, on ENaC current (C) and SGK1 phosphorylation (D). Cells were adapted to 1 mM as in (A) and VU0134992, or vehicle was added to both basolateral and apical media 5 min before changing \([K^+]\). Data are presented as means ±SEM. *\(P=0.0036\), **\(P=0.001\) represent the differences are significant by multiple paired two-tailed Student’s T-test with Bonferroni’s post corrections. NS, Not significant.
Figure 8. Role of WNK1 on extracellular [K+] stimulated phosphorylation of SGK1. (A) Western Blot analyses showing effects of WT (1-491) vs. Kinase dead WNK1 mutant (1-491) (K233M) on extracellular [K+] stimulated phosphorylation of SGK1. WNK1-deficient HEK-293 cells were transfected with FLAG-SGK1 and WNK1 (1-491) WT or (1-491) K233M. Cells were adapted to 1 mM [K+], and then media [K+] was either raised to 5 mM or kept at 1 mM for 1 h. In cells lacking WT or WNK1 K233M, raising extracellular [K+] to 5 mM failed to stimulate SGK1 phosphorylation. Cells expressing K233M exhibited higher abundance of pSGK1 compared to cells expressing WT WNK1. Data are presented as means ±SEM from three independent experiments. **P<0.01, ****P<0.0001 by one-way ANOVA. NS, Not significant.
Figure 9. Model of Na⁺ and K⁺ transport regulation by local and systemic [K⁺] in PCs of the CCD. Cellular model of the integrated regulation of ENaC activity, and ENaC-dependent K⁺ secretion, in response to altered plasma K⁺. Green arrows indicate stimulatory effects. Red arrow indicates inhibitory effect. Solid arrows indicate rapid direct effects (seconds to minutes), dashed arrows indicate slower genomic effects (minutes to hours). According to this scheme, elevation of basolateral [K⁺] triggers membrane depolarization by altering K⁺ channel-dominated resting potential, which with a possible involvement of intracellular [Cl⁻] and WNK1 stimulates mTORC2-dependent SGK1 activation via HM-phosphorylation. Activated SGK1 stimulates ENaC predominately by inhibiting Nedd4-2, but also potentially through other effects including ENaC phosphorylation. The ensuing increase in electrogenic Na⁺ transport enhances K⁺ secretion via K⁺ channels, predominantly renal outer medullary K channels (ROMK), but also large-conductance Ca₂-activated K (BK) channels. According to the model, SGK1 acts as a signal integrator, responding both to local signals that increase its activity, and to systemic hormonal signals (primarily aldosterone), which increase its expression.
Table 1. Urinary parameters in male NMRI and female C57/Bl6 mice.

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Gavage Treatment</th>
<th>NMRI ctrl vehicle</th>
<th>NMRI ctrl Benza</th>
<th>NMRI KCl vehicle</th>
<th>NMRI KCl Benza</th>
<th>C57Bl/6 ctrl vehicle</th>
<th>C57Bl/6 KCl vehicle</th>
<th>C57Bl/6 KCl Benza</th>
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<tbody>
<tr>
<td>N</td>
<td>6</td>
<td>6</td>
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<td>11</td>
<td>9</td>
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<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Female</td>
<td>Female</td>
<td>Female</td>
<td>Female</td>
</tr>
<tr>
<td>Body mass (g)</td>
<td>40.4±0.8</td>
<td>39.5±2.4</td>
<td>39.7±0.9</td>
<td>40.0±1.1</td>
<td>28.3±0.7</td>
<td>28.6±1.0</td>
<td>26.6±0.8</td>
<td>23.3±0.7</td>
</tr>
<tr>
<td>Net Na⁺ excretion (µmol/g BW/4h)</td>
<td>1.3±0.4</td>
<td>3.0±0.6*</td>
<td>3.6±0.6$</td>
<td>9.2±0.7*,$</td>
<td>1.6±0.3</td>
<td>4.0±0.4*</td>
<td>4.8±0.4$</td>
<td>10.9±1.4*,$</td>
</tr>
<tr>
<td>Net K⁺ excretion (µmol/g BW/4h)</td>
<td>1.8±0.4</td>
<td>0.2±0.05*</td>
<td>9.5±1.2$</td>
<td>1.9±0.2*,$</td>
<td>1.4±0.3</td>
<td>0.6±0.1*</td>
<td>10.6±0.8$</td>
<td>2.0±0.3*,$</td>
</tr>
<tr>
<td>Urinary excretion Na⁺/K ratio</td>
<td>0.7±0.1</td>
<td>20.2±5.3*</td>
<td>0.4±0.1</td>
<td>4.9±0.6*,$</td>
<td>1.3±0.2</td>
<td>12.7±2.9*</td>
<td>0.5±0.03$</td>
<td>3.4±1.0*,$</td>
</tr>
<tr>
<td>Net diuresis (µl/g BW/4h)</td>
<td>32±3</td>
<td>38±2</td>
<td>28±6</td>
<td>34±8</td>
<td>42±9</td>
<td>26±9</td>
<td>41±10</td>
<td>35±6</td>
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<tr>
<td>Plasma [K⁺] 1h post gavage (mM)</td>
<td>3.8±0.4</td>
<td>4.1±0.5</td>
<td>6.8±0.7$</td>
<td>6.9±1.0</td>
<td>na</td>
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<tr>
<td>Plasma <a href="pg/ml">aldosterone</a></td>
<td>228±24</td>
<td>309±46</td>
<td>234±29</td>
<td>299±52</td>
<td>na</td>
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Mice were given either control or KCl gavage in combination with post treatment of vehicle or benzamil (benza) injection. After gavage, mice were placed in balance cages for 4 hours under with free access to water. Urine was collected, and urinary electrolytes and volume were measured. Data represent mean ± SEM; differences were determined by one-way ANOVA. * indicate p < 0.05 vs. benzamil treatment and respective vehicle control and $ indicate p < 0.05 between control vs. KCl gavage with same vehicle or benzamil treatment.