Purinergic Inhibition of ENaC Produces Aldosterone Escape

James D. Stockand,* Elena Mironova,* Vladislav Bugaj,* Timo Rieg,† Paul A. Insel,† Volker Vallon,† Janos Peti-Peterdi,‡ and Oleh Pochynyuk§

*Department of Physiology, University of Texas Health Science Center at San Antonio, San Antonio, Texas; †Departments of Medicine and Pharmacology, University of California, San Diego, San Diego, California; ‡Department of Physiology and Biophysics, University of Southern California, Los Angeles, Los Angeles, California; and §Department of Integrative Biology and Pharmacology, University of Texas Health Science Center at Houston, Houston, Texas

ABSTRACT

The mechanisms underlying “aldosterone escape,” which refers to the excretion of sodium (Na⁺) during high Na⁺ intake despite inappropriately increased levels of mineralocorticoids, are incompletely understood. Because local purinergic tone in the aldosterone-sensitive distal nephron downregulates epithelial Na⁺ channel (ENaC) activity, we tested whether this mechanism mediates aldosterone escape. Here, urinary ATP concentration increased with dietary Na⁺ intake in mice. Physiologic concentrations of ATP decreased ENaC activity in a dosage-dependent manner. P2Y2−/− mice, which lack the purinergic receptor, had significantly less increased Na⁺ excretion than wild-type mice in response to high-Na⁺ intake. Exogenous deoxycorticosterone acetate and deletion of the P2Y2 receptor each modestly increased the resistance of ENaC to changes in Na⁺ intake; together, they markedly increased resistance. Under the latter condition, ENaC could not respond to changes in Na⁺ intake. In contrast, as a result of aldosterone escape, wild-type mice had increased Na⁺ excretion in response to high-Na⁺ intake regardless of the presence of high deoxycorticosterone acetate. These data suggest that control of ENaC by purinergic signaling is necessary for aldosterone escape.


Renal sodium (Na⁺) excretion influences BP by affecting systemic Na⁺ balance. Consequently, negative feedback in response to changes in Na⁺ balance, perceived as changes in effective circulating volume (ECV) and BP, control renal Na⁺ handling. The fine-tuning of Na⁺ balance occurs in the aldosterone-sensitive distal nephron (ASDN), including the connecting tubule (CNT) and the collecting duct (CD). Here, Na⁺ reabsorption is highest when ECV and BP are low. Activity of the epithelial Na⁺ channel (ENaC) is limiting for discretionary Na⁺ reabsorption across the ASDN.1–7 As ECV declines, activity of the renin-angiotensin-aldosterone system (RAAS) increases with the mineralocorticoid aldosterone, stimulating channel activity to decrease Na⁺ excretion in correction of falling ECV. Aldosterone increases the number and activity of ENaC in the apical membrane.8–11 Under normal conditions, the opposite is also true; aldosterone and, thus, ENaC activity decline in response to elevations in BP.

The thiazide-sensitive Na-Cl co-transporter
(NCC) in the distal convoluted tubule (DCT) is also a target of aldosterone with the mineralocorticoid increasing NCC activity and possibly transporter abundance in the apical membrane of DCT cells.12–16 This increase in NCC activity decreases Na\(^+\) excretion. Thus, aldosterone promotes distal nephron Na\(^+\) reabsorption by increasing the activity of ENaC and NCC.

The loss of negative-feedback regulation of renal Na\(^+\) excretion mediated by ENaC and NCC leads to hypertension. For instance, gain-of-function mutations in ENaC cause the channel to be hyperactive irrespective of mineralocorticoid status and systemic Na\(^+\) balance, thus leading to inappropriate Na\(^+\) retention and hypertension; conversely, loss-of-function mutations in ENaC can cause decreases in BP and renal salt wasting.2,3,5,7,17–19

The aldosterone-promoted decrease in renal Na\(^+\) excretion is overridden in some circumstances.20,21 This disruption of aldosterone action is termed aldosterone escape and results in avid Na\(^+\) excretion during high Na\(^+\) intake despite elevated mineralocorticoid levels. Aldosterone escape seems to be a protective mechanism to allow appropriate response to positive Na\(^+\) balance despite inappropriate mineralocorticoid levels. Such escape is important clinically, for example, in primary aldosteronism, in which it may ameliorate, to some degree, the hypertensive effects of high circulating levels of aldosterone.22–24 The mechanism allowing aldosterone escape is uncertain.

Aldosterone escape is known to be dependent on increases in renal vascular perfusion pressure rather than systemic factors, including changes in the levels of circulating hormones, such as renin and aldosterone, or renal nerve activity.25 This led to the idea that aldosterone escape is a manifestation of a pressure natriuresis response.13,25,26 This is associated with declining Na\(^+\) reabsorption in the distal nephron despite elevated levels of aldosterone and occurs independent of changes in GFR.26,27 Indeed, early micropuncture measurements demonstrated increases in urine flow and Na\(^+\) delivery to the CD during escape.26,27 Details about the specific site(s) along the distal nephron involved in decreased Na\(^+\) reabsorption during aldosterone escape and the exact transport proteins involved in this escape, though, largely remain obscure.

A study by Wang et al.13 revealed that during aldosterone escape, NCC levels in the DCT decrease. This response was selective because the levels of other apical membrane transport proteins, including ENaC, were unaffected. This finding supports the idea that NCC is, at least, one target for regulatory processes that mediate aldosterone escape, whereby decreasing NCC abundance facilitates Na\(^+\) excretion despite high levels of mineralocorticoid. The cellular mechanism underpinning declines in NCC levels during aldosterone escape is unknown. Similarly, it is unclear whether other transport proteins are involved in aldosterone escape because Na\(^+\) reabsorption is a manifestation not only of the number of transport proteins in the apical membrane but also of their activity.

Because ENaC is critical to aldosterone regulation of Na\(^+\) excretion, particularly in response to changes in Na\(^+\) balance, evidenced by the hypertension associated with gain of ENaC function,3,5,7 we sought to investigate the role of this channel in aldosterone escape. We recently demonstrated that local purinergic tone in the ASDN exerts paracrine downregulation of ENaC activity specifically by affecting channel open probability.28 This paracrine pathway is physiologically relevant because mice lacking the purinergic receptor P2Y2, responsible for the bulk of paracrine regulation in this system, have facilitated Na\(^+\) reabsorption in the distal nephron and mild increases in BP.29 Increases in NKCC2 and ENaC activity contribute to this elevated BP.11 It does not seem a coincidence that aldosterone and P2Y2 signaling target the same final effector proteins, possibly allowing one to compensate for the loss of the other. This idea suggests that purinergic regulation of ENaC may contribute to aldosterone escape, a possibility supported by another recent finding from our laboratories: Elevated BP in P2Y2\(^{-/}\) mice is not salt sensitive in the presence of normal-feedback regulation by RAAS but becomes salt sensitive and associated with inappropriately active ENaC when negative-feedback regulation by aldosterone and local purinergic signaling both are disrupted.11,29

To test the role of ENaC and its regulation by mineralocorticoids and purinergic signaling in aldosterone escape and to understand better the mechanism underpinning escape, we quantified the actions of mineralocorticoid on renal Na\(^+\) excretion, ENaC activity, and urinary ATP levels in wild-type (WT) and P2Y2\(^{-/}\) mice stressed with different dietary Na\(^+\) regimens. We found that urinary [ATP] increases with dietary Na\(^+\) intake such that physiologic [ATP] decrease ENaC activity, resulting in increased Na\(^+\) excretion. Increased Na\(^+\) excretion is greater in WT compared with P2Y2\(^{-/}\) mice and associated with an inability of ENaC, particularly when mineralocorticoid is clamped at high levels, to respond appropriately to changes in dietary Na\(^+\) intake in the latter animals. Because ENaC activity normally is sensitive to changes in Na\(^+\) balance even when mineralocorticoids are clamped at high levels, these results show that control of ENaC by purinergic signaling is necessary for complete aldosterone escape, consistent with the loss of aldosterone-escape in P2Y2\(^{-/}\) mice and their pronounced hypertension relative to normal mice in the presence of elevated mineralocorticoids and high Na\(^+\) intake.

RESULTS

ATP Levels in Urine Reflect Systemic Na\(^+\) Levels

To test for a cause-and-effect relation between systemic Na\(^+\) balance and urinary [ATP], we measured ATP levels in urine harvested directly from the bladders of WT mice maintained on nominally Na\(^+\)-free (<0.01%), regular-Na\(^+\) (0.32%), and high-Na\(^+\) (2%) diets. As summarized in Figure 1A, elevations in dietary Na\(^+\) significantly increased urinary ATP concentrations from a minimum of 7.9 ± 1.2 nM (n = 20) on a Na\(^+\)-free diet to 15.2 ± 3.3 (n = 11) and 50 ± 16 nM (n = 9) for 0.32 and 2% [Na\(^+\)] diets, respectively (P < 0.05). To exclude the possi-
We previously demonstrated that high μM concentrations of ATP act via apical membrane P2 receptors to decrease ENaC activity dramatically by reducing P_o. However, it is unclear whether physiologic ATP levels in urine (Figure 1) are sufficient to affect ENaC activity. Thus, we defined the concentration dependence of ATP inhibition of murine ENaC in the isolated ASDN by assessing the decrease in P_o. Figure 2A shows a representative gap-free current recording and documents ENaC activity in a cell-attached patch on the apical membrane of a principal cell in a freshly isolated, split-open murine ASDN before and after application of 10 μM ATP. ATP rapidly and markedly decreased ENaC P_o. The concentration-response curve for this inhibitory effect of ATP on ENaC activity (Figure 2B) yielded an IC_{50} of 33 ± 2 nM, a concentration in the physiologic range of ATP in urine (Figure 1A), and that is consistent with P2Y_2 receptor activation. 

We now show that a similarly marked decrease in ENaC P_o is seen in ASDN isolated from mice lacking Cx30. At our previous observation that urinary [ATP] is positively correlated with dietary Na^+ intake, 11 thus, these results support evidence that Cx30 expression contributes to ATP release into the CD and that the likely site for regulation of salt-sensitive ATP release into the urine is in the ASDN.

**Physiologic Concentrations of ATP (and UTP) Rapidly Decrease ENaC Activity in the Native Murine ASDN**

**Figure 2.** Physiologic concentrations of ATP and UTP rapidly decrease ENaC activity. (A) Representative gap-free current trace from a cell-attached patch on a principal cell in a split-open murine ASDN monitoring ENaC activity before and after addition of 10 μM ATP. Dashed lines show respective current levels with C denoting the closed state. Areas under the bars labeled 1 (control) and 2 (after ATP) are shown below with an expanded time scale. (B) Summary graph of dosage-dependent inhibition of ENaC activity by ATP (black) and UTP (gray). The number of paired experiments, similar to that in A, is shown for each concentration.
inhibitory purinergic regulation of ENaC.\textsuperscript{28} Unlike ionotropic P2X receptors and other P2Y receptors, the P2Y\textsubscript{2} receptor is activated equally well by the purine ATP and the pyrimidine UTP.\textsuperscript{30} Thus, we also assessed the dosage dependence of UTP actions on ENaC activity in the isolated, split-open murine ASDN (Figure 2B) and found that UTP inhibits ENaC activity as well as ATP, yielding an IC\textsubscript{50} of 49 ± 2 nM. These findings demonstrate that activators of P2Y\textsubscript{2} receptors at concentrations (in the case of ATP) found in the urine rapidly and markedly decrease ENaC activity in its native environment with the [ATP] associated with a high-Na\textsuperscript{+} urine rapidly and markedly decrease ENaC activity in its native environment with the [ATP] associated with a high-Na\textsuperscript{+} diet inhibiting ENaC activity by at least 50% whereas that associated with a low-Na\textsuperscript{+} diet has little inhibitory effect on the channel.

Disruption of Local Control of ENaC by Purinergic Tone in P2Y\textsubscript{2}−/− Mice Impairs Renal Na\textsuperscript{+} Excretion

We next assessed the physiologic consequences on renal Na\textsuperscript{+} excretion of negative regulation of ENaC by the purinergic system intrinsic to the ASDN, which is responsive to changes in Na\textsuperscript{+} intake as shown in the previous section. For these purposes, we measured and compared plasma Na\textsuperscript{+} concentrations (P\textsubscript{Na}), shown in Figure 3A, and urinary Na\textsuperscript{+} concentrations ([U\textsubscript{Na}]), shown in Figure 3B, for WT and P2Y\textsubscript{2}−/− mice maintained on low-, regular-, and high-Na\textsuperscript{+} regimens. After 1 week on a given Na\textsuperscript{+} diet, P\textsubscript{Na} varied little in either WT or P2Y\textsubscript{2}−/− mice with plasma Na\textsuperscript{+} concentrations in the latter mice not differing from those in WT mice under any feeding condition tested. In contrast to the findings for P\textsubscript{Na}, U\textsubscript{Na} showed a significant dependence on the feeding regimen for both WT and P2Y\textsubscript{2}−/− mice; U\textsubscript{Na} was greatest in mice maintained on a high-Na\textsuperscript{+} diet. With the Na\textsuperscript{+}-deficient diet, U\textsubscript{Na} was not significantly different between WT and P2Y\textsubscript{2}−/− mice (6.1 ± 1.7 versus 4.9 ± 1.0 mM). In contrast, U\textsubscript{Na} was significantly lower for P2Y\textsubscript{2}−/− compared with WT mice maintained with regular- and high-Na\textsuperscript{+} diets (96 ± 15 versus 124 ± 13 mM and 265 ± 16 mM versus 352 ± 30 mM, respectively; \textit{P} < 0.05), although both WT and P2Y\textsubscript{2}−/− mice had salt-dependent increases in U\textsubscript{Na}. These findings are consistent with P2Y\textsubscript{2}−/− mice having a diminished capacity to excrete Na\textsuperscript{+}.

Figure 3C summarizes the effects of varying systemic Na\textsuperscript{+} levels and disruption of the local purinergic system in the ASDN through genetic deletion of the P2Y\textsubscript{2} receptor on relative Na\textsuperscript{+} excretion ([U\textsubscript{Na}] [mM]/[U\textsubscript{cre} [mg/dl]). U\textsubscript{cre} did not differ between WT and P2Y\textsubscript{2}−/− mice and was unaffected by dietary Na\textsuperscript{+} intake (data not shown). As expected, little Na\textsuperscript{+} was excreted by either group under salt-restricted conditions. In contrast, relative Na\textsuperscript{+} excretion rose in mice fed normal- and high-Na\textsuperscript{+} diets. In these latter conditions, relative Na\textsuperscript{+} excretion was significantly lower in P2Y\textsubscript{2}−/− compared with WT mice. Thus, disrupting negative regulation of ENaC by compromising the local purinergic system significantly impairs an appropriate increase in Na\textsuperscript{+} excretion in response to increased Na\textsuperscript{+} intake.

Compensation by the Local Purinergic System Allows for Aldosterone Escape

Although impaired in the absence of normal regulation of ENaC by local purinergic signaling, Na\textsuperscript{+} excretion in P2Y\textsubscript{2}−/− mice retained a dependence on salt intake. This most likely reflects, in part, the contribution of normal feedback regulation of ENaC in ASDN by the mineralocorticoid system responding to increases in Na\textsuperscript{+} intake. Other nephron segments also contribute to proper Na\textsuperscript{+} excretion in response to elevated Na\textsuperscript{+} intake, other signaling inputs in addition to mineralocorticoids and purinergic signaling may influence ENaC activity in response to changes in systemic Na\textsuperscript{+} intake, and ATP may target effectors other than ENaC in response to changes in Na\textsuperscript{+} balance. To predict the involvement of ENaC to Na\textsuperscript{+} excretion in response to increases in salt intake and to identify the contributions of salt-dependent mineralocorticoid and local purinergic regulation of this channel to salt excretion, we developed an index to gauge the responsiveness of ENaC to changes in Na\textsuperscript{+} balance. We measured ENaC activity in the isolated split-open murine ASDN with patch-clamp electrophysiology, as in Figure 2A, from mice treated with nominally free- and high-Na\textsuperscript{+} diets, and then divided the activity observed on a high-Na\textsuperscript{+} diet by that on a Na\textsuperscript{+}-free diet. As we showed previously,\textsuperscript{10,11} ENaC activity is normally very responsive to salt intake because ENaC activity decreases and increases in response to high and low salt intake, respectively, through the cumulative actions of feedback regulation by the mineralocorticoid system, local purinergic signaling, and other inputs.\textsuperscript{10,11,28} As shown in Figure 4, fractional ENaC activity in WT mice, in the absence of any perturbation other than changing salt feeding, is low, indicating a high degree of responsiveness to changes in Na\textsuperscript{+} balance. Raw ENaC activity data for WT mice maintained with nominally Na\textsuperscript{+}-free and high-Na\textsuperscript{+} feeding in the absence and presence of deoxycorticosterone acetate (DOCA) are shown and compared in Table 1 (some of these data were
DISCUSSION

Our results are consistent with the presence of local regulation of ENaC by a purinergic system intrinsic to the ASDN that enables, at least in part, aldosterone escape: Elevated Na⁺ excretion in the presence of high levels of mineralocorticoid. This purinergic regulation is mediated by P2Y₂ receptors and presumably by the increase in the urinary levels of purinergic agonists (ATP and/or UTP) that accompanies increased dietary...
Na\textsuperscript{+} intake and results in decreases in ENaC activity and increases in Na\textsuperscript{+} excretion. Loss of proper feedback control, by disrupting both mineralocorticoid and purinergic regulation, uncouples ENaC from responding normally to changes in systemic Na\textsuperscript{+}. This affects regulation of BP in P2Y\textsubscript{2}\textsuperscript{-/-} mice with these mice, in contrast to WT mice, having pronounced elevations in BP in the presence of high salt intake and high levels of DOCA because ENaC is no longer responsive to changes in Na\textsuperscript{+} balance.

We did not investigate the interplay between regulation of ENaC by local purinergic signaling during aldosterone escape along with other factors and targets, such as ANP, nitric oxide signaling, and NCC, that may be involved in such escape.\textsuperscript{13,36} Rather, our goal was to assess the role played by local purinergic regulation of ENaC in aldosterone escape and to understand the consequences of loss of purinergic regulation during the escape process.

Our observation that urinary [ATP] and [UTP] increase as a function of dietary Na\textsuperscript{+} intake suggests a tight link between systemic Na\textsuperscript{+} balance and release of ATP and UTP into the urine, although the specific mechanisms for this relationship remain obscure (see also reference 11). Moreover, which nephron segments serve as the primary source of salt-sensitive ATP release are not known. Whether ATP versus UTP or some other purinergic receptor agonist is the primary physiologic stimulus of P2Y\textsubscript{2} receptors during ENaC regulation also is obscure. Here, we followed ATP as a representative P2Y\textsubscript{2} agonists found in urine. Some released ATP must arise from the ASDN because we see autocrine/paracrine (tonic) regulation of ENaC via apical P2Y\textsubscript{2} receptors in this nephron segment when it is isolated from the rest of the kidney.\textsuperscript{11,28} Elevation of systemic Na\textsuperscript{+} increases plasma and urine volume and Na\textsuperscript{+} content, as well as urine flow, and can influence tubular cell volume. It is not clear which of these is the proximate trigger for ATP release into the urine: Both changes in tubular flow rate and cell volume have been reported to promote tubular ATP release.\textsuperscript{34-39}

Our findings with Cx30\textsuperscript{-/-} mice and data for ATP release in these animals performed previously\textsuperscript{33,34} provide evidence that Cx30\textsuperscript{-/-} in the distal nephron, particularly in intercalated cells, is important for ATP release into the urine. Our finding that the bulk of salt-sensitive ATP release is lost in Cx30\textsuperscript{-/-} mice suggests, in addition, that this route of release may be particularly important during responses to changes in Na\textsuperscript{+} balance. This idea is consistent with the findings that renal Na\textsuperscript{+} excretion and pressure natriuresis are impaired in Cx30\textsuperscript{-/-} mice and that these mice develop ENaC-dependent, salt-sensitive elevations in BP.\textsuperscript{34} Principal cells in the ASDN are another possible source of ATP release with release from these cells seeming to depend on the presence of the apical primary cilia.\textsuperscript{37,40} Interestingly, this monocilia may be involved in sensing urine flow, which rises upon increases in systemic Na\textsuperscript{+} levels. Other possible sources of urinary ATP potentially capable of affecting transport proteins in the distal nephron are upstream nephron segments, including the thick ascending limb.\textsuperscript{38,39} For instance, ATP released from the thick ascending limb in response to flow acts in a paracrine manner to mobilize Ca\textsuperscript{2+} in tubules from WT but not P2Y\textsubscript{2}\textsuperscript{-/-} mice.\textsuperscript{59}

Although the precise mechanisms that trigger ATP release into the urine in response to elevations in Na\textsuperscript{+} intake and the intrarenal source of this paracrine-acting ATP remain to be definitively established, the observation that increases in Na\textsuperscript{+} balance increase urinary [ATP] is consistent with previous findings.\textsuperscript{11,30,31} Micropuncture studies have shown that ATP levels in the ultrafiltrate of the proximal tubule climb as high as 300 nM with distal tubule levels being approximately 3.5-fold lower.\textsuperscript{41} In agreement with this, we found urinary ATP levels in the nanomolar range. As a result of the actions of ecto-ATPase and ecto-5’-nucleotidase and other extracellular enzymes capable of metabolizing ATP, quantification of [ATP] from urine in the bladder may underestimate, somewhat, actual values. Alternatively, water reabsorption distal to the site of ATP release may concentrate yielding ATP values modestly higher in the final urine compared with urine in the ASDN. Nevertheless, [ATP] concentrations consistent with those measured in urine have a marked effect on ENaC activity in its native environment in isolation from possible secondary effects, such as changes in perfusion pressure, urine flow, and other factors that may complicate interpretation. Thus, the [ATP] associated with high Na\textsuperscript{+} levels causes marked inhibition of ENaC, whereas the channel is uninhibited with [ATP] at lower levels of dietary Na\textsuperscript{+}, results consistent with the regulation of ENaC by ATP signaling in the ASDN playing an important role in the renal Na\textsuperscript{2+} excretory response to changes in systemic Na\textsuperscript{+} levels. Our findings showing that genetic deletion of the P2Y\textsubscript{2} receptor, which is the primary target for paracrine ATP regulation of ENaC in the ASDN,\textsuperscript{11,28} impairs excretion of Na\textsuperscript{+}
particularly during elevations of Na\(^+\) intake are in agreement with this idea. Our findings also agree with previous results showing that renal Na\(^+\) excretion is impaired in P2Y\(_2\)/−/− mice.\(^{29}\)

Together, our results demonstrate that increasing systemic Na\(^+\) increases urinary [ATP] to levels capable of suppressing ENaC activity and facilitating Na\(^+\) excretion. RAAS has a similar role in mediating an ENaC response to rinsergic signaling provides a mechanism for escaping the purinergic and mineralocorticoid signaling uncouples ENaC thereby facilitating Na\(^+\) excretion. ENaC activity thus changes inversely with respect to changes in systemic Na\(^+\) levels.

P2Y\(_2\)/−/− mice have facilitated renal Na\(^+\) reabsorption associated with suppressed plasma renin and aldosterone levels and lower-than-normal plasma K\(^+\).\(^{29}\) The latter is because K\(^+\) is secreted in the distal nephron to maintain charge neutrality as Na\(^+\) is reabsorbed via ENaC. This profile along with salt-sensitive changes in Na\(^+\) excretion in BP is the clinical manifestation of Liddle's syndrome: Gain of ENaC function.\(^{5,7,17}\) BP in P2Y\(_2\)/−/− animals, in the absence of any other perturbation, however, is salt resistant.\(^{29}\) This suggests little involvement of ENaC; however, on the basis of our previous work\(^{11,28}\) and results of this study, we conclude that regulation of ENaC by ATP functions in parallel and complements regulation by aldosterone. This means that ENaC activity can change, to some degree, in response to changes in Na\(^+\) intake when one but not both salt-sensing control systems is lost. In other words, ENaC activity, as shown in Figure 4, retains its ability to respond to changes in Na\(^+\) intake in the absence of mineralocorticoid or purinergic regulation but not when both are absent. Likewise, renal Na\(^+\) handling and BP are mostly normal in the absence of either but not of both salt-sensing regulatory mechanisms that govern ENaC activity. Consistent with this idea are our findings showing that relative Na\(^+\) excretion, although modestly lower in P2Y\(_2\)/−/− mice compared with WT mice, retains most of its dependence on Na\(^+\) balance and that Na\(^+\) excretion remains elevated during aldosterone escape despite elevated mineralocorticoid levels. In contrast, simultaneously disrupting regulation by both purinergic and mineralocorticoid signaling uncouples ENaC from being able to respond appropriately to Na\(^+\), giving it a high salt resistance and driving Na\(^+\) excretion to inappropriately low levels in the presence of high salt intake (see Figure 5).

The impaired ability of ENaC to respond to changes in Na\(^+\) intake leads to the salt-sensitive hypertension of P2Y\(_2\)/−/− mice treated with DOCA. As a result of aldosterone escape, WT mice treated with DOCA have BP that is not salt sensitive. We conclude that inhibition of ENaC by local purinergic signaling provides a mechanism for escaping the actions of aldosterone.

**CONCISE METHODS**

All chemicals and materials were from Sigma (St. Louis, MO) unless noted otherwise and were of reagent grade. Animal use and welfare adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals following a protocol reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio. For experiments, WT (C57BL/6), P2Y\(_2\)/−/− (backcrossed and inbred into the C57BL/6 background; described in detail previously\(^{11,28,29}\)) and Cx30\(^+/−\) mice were maintained on a nominally Na\(^+\)-free diet (<0.01% Na\(^+\); Harlan TEKLAD TD.90228), regular-Na\(^+\) diet containing 0.32% Na\(^+\) (Harlan TEKLAD TD.7912), or a high-Na\(^+\) diet (2% Na\(^+\); Harlan TEKLAD TD.92034) 1 week before experimentation. For some experiments, mice were administered a subcutaneous injection of 2.4 mg of DOCA dissolved in 150 μl of olive oil for 3 consecutive days before being killed.

Isolation of the ASDN containing CNT and CD suitable for electrophysiology has been described previously.\(^{10,11,28}\) Briefly, mice were killed by CO\(_2\) administration followed by cervical dislocation. Kidneys were immediately removed and were cut into thin slices (<1 mm), which were placed into ice-cold physiologic saline solution buffered with HEPES (pH 7.4). The ASDN was identified as merging of CNT into CD and was mechanically isolated from cortical sections of kidney slices by microdissection using watchmaker forceps under a stereomicroscope. Isolated ASDN was allowed to settle onto 5 × 5-mm coverglass coated with poly-l-lysine. Coverglass containing ASDN was placed in a perfusion chamber mounted on an inverted Nikon Eclipse TE2000 microscope and superfused with room temperature HEPES-buffered (pH 7.4) saline solution. ASDN were split open with two sharpened micropipettes controlled with different micromanipulators to gain access to the apical membrane and were used within 1 to 2 hours of isolation.

ENaC activity in principal cells of murine ASDN was determined in cell-attached patches on the apical membrane made under voltage-clamp conditions (−V\(_m\) = −60 mV) using standard procedures.\(^{10,28}\) Current recordings were made in a still bath with experimental reagents added directly to the recording chamber. Recording pipettes had resistances of 10 to 15 MΩ. Typical bath and pipette solutions were as follows: 150 mM NaCl, 5 mM KCl, 1 mM CaCl\(_2\), 2 mM MgCl\(_2\), 5 mM glucose, and 10 mM HEPES (pH 7.4) and 140 mM LiCl, 2 mM MgCl\(_2\), and 10 mM HEPES (pH 7.4), respectively. For each experimental condition, ASDN from at least three mice were assayed. Gap-free single-channel current data from gigahm seals were acquired (and subsequently analyzed) with an Axopatch 200B (Axon Instruments) or EPC-9 (HEKA Instruments) patch-clamp amplifier interfaced via a Digidata 1322A (Axon Instruments) to a PC running pClamp 9.2 software (Axon Instruments). Currents were low-pass-filtered at 100 Hz with an eight-pole Bessel filter (Warner Instruments). Unitary current (i) was determined, as normal, from all-point amplitude histograms fitted with single- or multi-Gaussian curves using the standard 50% threshold criterion to differentiate between events. Events were inspected visually before acceptance. Channel activity, defined as \(NP_\alpha\), was calculated using the equation \(NP_\alpha = (t_1 + 2t_2 + \ldots + nt_n)\), where \(N\) and \(P_\alpha\) are the number of ENaC in a patch and the mean open probability of these channels, respectively, and \(t_n\) is the fractional open time spent at each of the ob-
served current levels. \( P_o \) was calculated by dividing \( N P_o \) by the number of active channels within a patch as defined by all-point amplitude histograms. For calculating \( P_o \) in paired experiments, \( N \) was fixed as the greatest number of active channels observed in control or experimental conditions. In such paired patch-clamp experiments, \( N \) cannot change in response to the experimental maneuver (e.g., ATP), so any detected effect must be an effect on \( P_o \). The error associated with calculating \( P_o \) increases as this variable moves away from 0.5 and approaches 0 or unity. To ensure reliable calculation of \( P_o \), we measured \( P_o \) with standard and accepted tools using long recording times (>1 minute) and patches containing five or fewer channels. This approach, which provides the most confidence other than using seals with only one channel, is routinely used to determine \( P_o \). The frequency (f) of observing ENaC in a patched membrane for a given condition was calculated by dividing the number of seals containing at least one active channel for that condition by the total number of gigaseal seals formed under that condition.

Urine and blood (on 8 U heparin/500 μl serum) samples were collected directly from the bladder and heart, respectively, immediately after mice were killed. Heparinized blood samples were centrifuged at 1000 rpm for 40 minutes to separate plasma from blood cells. Urinary and plasma Na\(^+\) concentrations were quantified in fresh samples using a PFP7 flame photometer (Techne, Burlington, NJ). Relative Na\(^+\) excretion (\( U_{Na}/U_{cre} \)) was calculated by dividing [\( U_{Na} \)] (in mM) by [\( U_{cre} \)] (in mg/dl). Creatinine values were assessed with an improved Jaffe reaction (two-tailed paired \( t \) test). Data from different experiments were compared with a Student’s \( t \) test. Data from before and after treatment within the same experiment were compared with the paired \( t \) test. Data from different experiments were compared with a (two-tailed) \( t \) test or an one-way ANOVA using the Dunnett posttest comparing treatment groups with a single control group (Na\(^+\)-free diet). \( P \leq 0.05 \) considered significant. For presentation, current data from before and after treatment conditions are presented as mean \( \pm \) SEM. Data from before and after treatment within the same experiment were compared with the paired \( t \) test. Data from different experiments were compared with a (two-tailed) \( t \) test or an one-way ANOVA using the Dunnett posttest comparing treatment groups with a single control group (Na\(^+\)-free diet). \( P \leq 0.05 \) considered significant. For presentation, current data from some cell-attached patches were subsequently software filtered at 50 Hz and slow baseline drifts were corrected.

ACKNOWLEDGMENTS

This research was supported by National Institutes of Health grants R01DK59594 (to J.D.S.), R01DK56248, R01DK28602, and R01HL94728 (to V.V.), and R01GM66232 (to P.A.I.); American Heart Association (AHA) Establish Investigator Awards 0640054N (to J.D.S.) and 0640056N (to J.P.-P.); AHA Grant in Aid 10GRNT3440038 (to V.V.); AHA SDG2230391 (to O.P.); German Research Foundation (RI1535/3-1 and 3-2 to T.R.); a National Kidney Foundation Fellowship (to T.R.); and the Research Service of the Department of Veterans Affairs (to V.V.).

DISCLOSURES

None.

REFERENCES


