Activation of the epithelial Na\(^+\) channel in the collecting duct by vasopressin contributes to water reabsorption

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**Am J Physiol Renal Physiol 297:** F1411–F1418, 2009. First published August 19, 2009; doi:10.1152/ajprenal.00371.2009.—We used patch-clamp electrophysiology on isolated, split-open murine collecting ducts (CD) to test the hypothesis that regulation of epithelial sodium channel (ENaC) activity is a physiologically important effect of vasopressin. Surprisingly, this has not been tested directly before. We ask whether vasopressin affects ENaC activity distinguishing between acute and chronic effects, as well as, parsing the cellular signaling pathway and molecular mechanism of regulation. In addition, we quantified possible synergistic regulation of ENaC by vasopressin and aldosterone associating this with a requirement for distal nephron Na\(^+\) reabsorption during water conservation vs. maintenance of Na\(^+\) balance. We find that vasopressin significantly increases ENaC activity within 2–3 min by increasing open probability (P\(_o\)). This activation was dependent on adenylyl cyclase (AC) and PKA. Water restriction (18–24 h) and pretreatment of isolated CD with vasopressin (∼30 min) resulted in a similar increase in P\(_o\). In addition, this also increased the number (N) of active ENaC in the apical membrane. Similar to P\(_o\), increases in N were sensitive to inhibitors of AC. Stressing animals with water and salt restriction separately and jointly revealed an important effect of vasopressin: conservation of water and sodium (Na\(^+\)) reabsorption from tubular fluid concentrating urine. The primary nephron, to increase water permeability facilitating water reabsorption from tubular fluid concentrating urine. The primary nephron, to increase water permeability facilitating water reabsorption from tubular fluid concentrating urine.

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The activity of the epithelial Na\(^+\) channel (ENaC), which like AQP2 is localized to the luminal plasma membrane of principal cells, is limiting for discretionary Na\(^+\) reabsorption across the distal nephron, including the connecting tubule and collecting duct (5, 18, 23, 29, 51). ENaC is blocked by the K\(^+\)-sparing diuretic amiloride, and as the limiting step in Na\(^+\) reabsorption and a target of the adrenal corticosteroid aldosterone, serves as a critical end effector of the negative feedback homeostatic control system, the renin-angiotensin-aldosterone system (RAAS), governing plasma [Na\(^+\)] and blood pressure (5, 23, 51). The importance of ENaC and its proper regulation to renal Na\(^+\) handling and blood pressure is clear when considering the inappropriate salt retention and hypertensive phenotypes associated with ENaC gain of function, and the renal salt wasting and hypotension associated with its loss of function (5, 23, 28).

Several lines of evidence support contribution of discretionary Na\(^+\) reabsorption mediated by ENaC to AVP-mediated water reabsorption. Indeed, AVP has long been recognized to possess antinatriuretic actions along with its better described antidiuretic actions (1, 3, 12, 17, 19, 21, 24, 27); although it is not clear whether this antinatriuretic effect contributes to or results from the antidiuretic actions of the hormone. Strong indication that ENaC may be involved in a physiologically important AVP response first arose from findings that in addition to increasing water permeability, AVP also increases transepithelial voltage, decreases transepithelial resistance, and increases amiloride-sensitive, unidirectional luminal-to-basolateral Na\(^+\) fluxes in isolated perfused rat collecting ducts and other models of the mammalian collecting duct (11, 17, 20, 21, 34, 36, 37, 42, 48). In addition, aldosterone and AVP have additive effects on sodium flux suggesting synergistic action on a common target (11, 20, 37). From such results, it became widely accepted that AVP through its cognate signaling pathway increases ENaC activity in principal cells of the mammalian collecting duct, although this has yet to be specifically tested.

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Additional support for the idea that AVP increases ENaC activity comes from findings where AVP increases the abundance of ENaC subunits in principal cells, and similar to its actions on AQP2, promotes trafficking and insertion of ENaC via cAMP signaling into the apical membrane of native principal cells and cell lines (13, 14, 32, 33, 39); although an AVP effect on ENaC trafficking has been challenged recently (39). AVP, in addition, has a robust effect on amiloride-blockable short-circuit current across cultured epithelial cells, and in the only study directly testing this to date, ENaC activity in immortalized renal epithelial cells (4, 9, 10, 15, 26, 32, 45). cAMP has similar actions on ENaC in culture cells and increases the Na⁺ conductance of the luminal membrane in isolated tubules (9, 30, 32, 41, 42); although it often has been difficult to achieve clear upregulation of ENaC function by AVP and cAMP using cloned channel subunits in expression systems (43).

While the preponderance of evidence supports AVP activation of ENaC in the mammalian collecting duct, this evidence results from indirect or extrapolated findings and as such, needs to be evaluated in the context of studies directly assessing AVP actions on the channel in its native surroundings. In the current study, we use patch-clamp electrophysiology to specifically test whether AVP modulates ENaC activity in the isolated, split-open murine collecting duct. We find that AVP increases ENaC activity increasing channel Pₒ in a rapid but sustained manner. Long-term exposure to AVP and water restriction results in a similar increase in Pₒ with a significant increase in the number of active channels in the apical membrane also appearing over time. Increases in Pₒ and N, as expected, require cAMP and PKA signaling. In addition, aldosterone and AVP were additive with ENaC most active in states of avid water and Na⁺ conservation. ENaC is active also during conditions such as high dietary sodium intake combined with water restriction that promote water but not sodium conservation, suggesting that AVP has a quantitatively important action on ENaC during water reabsorption uncoupled from the role played by the channel in Na⁺ balance.

METHODS

All chemicals were from BioMol (Plymouth Meeting, PA) and Sigma (St. Louis, MO) unless noted otherwise. [Arg⁸]-vasopressin was from MP Biomedicals (catalogue no. 152962; Solon, OH). Mice (C57BL/6) were from Charles River Laboratories (Wilmington, MA).

Collecting ducts were isolated from mice 2–3 mo old of either sex. Mice were maintained for at least 1 wk before collecting duct isolation on a fixed diet containing either 0.32% [Na⁺] (standard chow; Harlan Teklad TD.7912), <0.01% [Na⁺] (nominally Na⁺-free diet; TD.90228), or 2% [Na⁺] (high-Na⁺ diet; TD.92034). For some experiments, mice were water restricted for 18–24 h before collecting duct isolation.

Isolating collecting ducts suitable for electrophysiology have been described (7, 35, 49). In brief, kidneys were cut into thin slices (<1 mm) with slices placed in ice-cold physiologic saline solution (pH 7.4). Collecting ducts were mechanically isolated from these slices by microdissection using watchmaker forceps under a stereomicroscope. Isolated cortical collecting ducts were allowed to settle onto 5 × 5-mm coverglass coated with poly-L-lysine. Coverglass containing collecting ducts was placed within a perfusion chamber mounted on an inverted Nikon TE2000 microscope and superfused with a physiologic saline solution buffered with HEPES (pH 7.4). Collecting ducts were split open with sharpened micropipettes controlled with micro-manipulators to gain access to the apical membrane. Collecting ducts were used within 1–2 h of isolation. 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 Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio.

Cell-attached patches were made under voltage-clamp conditions (−Vp = −60 mV) on the apical plasma membranes of principal cells in isolated, split-open murine collecting ducts using standard procedures (7, 35, 49). Current recordings were made in a still bath with experimental reagents added sequentially to the recording chamber (final concentration calculated using bath volume). For experiments involving pretreatment, isolated collecting ducts were treated with reagent for ≥30 min before seal formation. Recording pipettes had resistances of 10–15 MΩ. Typical bath and pipette solutions were (in mM) 150 NaCl, 5 mM KCl, 1 CaCl₂, 2 MgCl₂, 5 glucose, and 10 HEPES (pH 7.4) and 140 LiCl, 2 MgCl₂, and 10 HEPES (pH 7.4), respectively. Gap-free single-channel current data from gigahm seals were acquired (and subsequently analyzed) with an EPC-9 (HEKA Instruments) patch-clamp amplifier interfaced with a PC running the pClamp 9.2 suite of software (Axon Instruments). Currents were low-pass filtered at 100 Hz and digitized at a sampling rate of 2 kHz. 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. All events were inspected visually before acceptance. Channel activity defined as Npₒ was calculated using the following equation: NPₒ = Σ(tₒ + 2tₒ + . . . + nₒ), where N and Pₒ are the number of ENaC in a patch and the mean open probability of these channels, and tₒ is the fractional open time spent at each of the observed current levels. Pₒ is calculated by dividing Npₒ by the number of active channels within a patch as defined by all-point amplitude histograms. For calculating Pₒ in paired experiments, N is fixed as the greatest number of active channels observed within either control or experimental condition. The error associated with calculating Pₒ increases as this variable moves away from 0.5 approaching 0 or unity (25, 30). To ensure reliable calculation of Pₒ, this variable was calculated in patches containing five or fewer channels and from recording periods greater than 1 min. The frequency of observing ENaC (f) for a given condition was equal to the number of patches containing at least one active channel divided by the total number of viable seals made for that condition. ENaC activity, Pₒ, N, and f for each condition were quantified from collecting ducts isolated from at least three different mice.

Summarized data are reported as means ± SE. 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 Student’s (2-tailed) t-test or a one-way ANOVA using the Dunnett posttest comparing treatment groups to a single control group. Differences in f were evaluated using a z-test comparing proportions between groups. For presentation, current data from some cell-attached patches were subsequently software filtered at 50 Hz and slow baseline drifts were corrected.

RESULTS

Figure 1A contains a representative current trace from a cell-attached patch formed on the apical membrane of a principal cell in a split-open murine collecting duct before and after addition of 1.0 μM AVP. This patch contains at least four ENaC. At this holding potential (−Vp = −60 mV) with our pipette and bath solutions, inward current is downwards. This collecting duct comes from a mouse maintained on standard chow. As clear in this representative experiment, AVP rapidly and markedly increases ENaC activity by increasing Pₒ. As summarized in Fig. 1B from 16 similar experiments, AVP
significantly increases ENaC $P_o$ from $0.30 \pm 0.04$ to $0.57 \pm 0.04$ within 2–3 min.

As shown in Fig. 2, adenylyl cyclase and PKA are required for the acute actions of AVP on ENaC $P_o$. Figure 2, A–C, summarizes data from paired experiments testing the effects of AVP on ENaC in collecting ducts pretreated first for at least 30 min with the AC inhibitor MDL-12 330A (100 μM; Fig. 2A) and the PKA inhibitors Rp-cAMPS (100 μM; Fig. 2B) and H-89 (20 μM; Fig. 2C). In the presence of inhibited AC and PKA, vasopressin had no effect on ENaC $P_o$. As shown in Fig. 2D, preinhibition of AC but not PKA, in addition to abolishing the stimulatory actions of AVP on ENaC $P_o$, also resulted in a marked and significant decrease in resting $P_o$. This suggests that AC signaling and production of cAMP are pivotal to both setting resting ENaC activity as well as conveying a stimulatory signal to the channel. PKA, in contrast, appears chiefly to be involved in conveying a stimulatory signal to the channel.

Similar to the acute actions of vasopressin on isolated collecting ducts, more prolonged manipulations of vasopressin and whole animal water levels influenced ENaC. The repre-
sentative ENaC current traces in Fig. 3A are from patches formed on principal cells from collecting ducts isolated from animals fed a normal-salt diet (0.32% [Na⁺]). Directly following isolation but before seal formation, these collecting ducts were pretreated for ~30 min with vehicle (control; top trace) and 1.0 μM AVP (middle trace). Also shown is a representative trace for ENaC (bottom trace) in a principal cell of a collecting duct isolated from an animal water restricted for 18–24 h (water restriction increased mean urinary osmolality to levels greater than 3,000 mosmol/kgH₂O often approaching 3,500–3,800 mosmol/kgH₂O). As is clear in these representative experiments and in the summary graph in Fig. 3B, chronic exposure of isolated collecting ducts to AVP had an effect similar to water deprivation with both increasing P₀ compared with control. Moreover, chronic exposure to exogenous AVP ex vivo significantly increased ENaC density. Water deprivation, similarly, resulted in ENaC density trending upwards. Like P₀, this effect on density was sensitive to inhibition of AC (see Supplemental Fig. S1; the online version of this article contains supplemental data).

To test the idea that active ENaC is critical to maintenance of water reabsorption and to arrive at a quantitative understanding of the possible interplay between AVP and aldosterone regulation of ENaC during physiological states of sodium and water conservation, we assessed ENaC activity in collecting ducts from mice maintained on high-Na⁺ diets and diets nominally free of sodium in the presence and absence of water restriction. Summary results from such experiments are shown in Fig. 4. We find, as shown in Fig. 4A and as expected, an inverse relationship between ENaC P₀ and the availability of dietary Na⁺. Importantly, transposed onto this, water restriction significantly increases ENaC P₀ regardless of the availability of sodium in the diet with P₀ being highest when animals are forced to conserve both water and salt supporting synergistic regulation of ENaC by AVP and aldosterone. We noted an interesting difference, as highlighted in Fig. 4B, when comparing the effects of dietary salt and water restriction on the density of ENaC within patched membranes. Strictly speaking, this is N (see METHODS). Again, as expected, we observed an inverse relationship between dietary Na⁺ availability and ENaC N with the mean number of ENaC increasing in patches from collecting ducts isolated from animals fed a nominally Na⁺-free diet compared with a high-Na⁺ diet. Water restriction had a subtler effect. Depriving animals of water for 18–24 h only modestly affected the mean number of ENaC within a patch under both low- and high-salt conditions, but as shown in Fig. 4C, had a significant effect on the frequency (f) of observing at least one active ENaC in membrane patches from animals fed a high-Na⁺ diet: a condition where aldosterone is expected to be suppressed but the animal is avidly attempting to conserve water. In other words, water deprivation did not increase the mean density of ENaC within membrane patches but did increase the chance of seeing at least one active ENaC when forming a seal. This means that water deprivation and associated increases in AVP increase apical membrane ENaC numbers by increasing the overall density of channels within the entire membrane but not necessarily by increasing the number of ENaC within any specific membrane cluster. Similar to water restriction, decreases in dietary Na⁺ also increased ENaC frequency with manipulation of either water or sodium independently being able to maximize ENaC frequency but with only low sodium (via aldosterone) being capable of maximizing both frequency and N. The net effect on overall ENaC activity (reported as fN₀), as shown in Fig. 4D, then, is that water restriction in complement with dietary Na⁺ restriction increases ENaC activity with activity increasing greatest during a physiological state requiring maximal water and sodium conservation.

The model presented in Fig. 5 emphasizes the independent and synergistic activities of AVP and aldosterone on ENaC. AVP and aldosterone both independently increase P₀ with sodium restriction via aldosterone having a quantitatively larger effect compared with water restriction via AVP. P₀ reaches a maximum under low dietary sodium intake and water restriction supporting synergy. Sodium and water restriction both also increase the frequency of ENaC in the apical membrane.
but either alone is capable of saturating this parameter. Finally, sodium restriction but not water restriction increases ENaC density within membrane clusters, suggesting that aldosterone but not AVP has a trophic effect in addition to more rapid effects on $P_\text{o}$ and channel trafficking.

**DISCUSSION**

We unequivocally demonstrate here for the first time that AVP specifically targets ENaC in the mammalian collecting duct to increase channel activity. The molecular mechanisms underlying this activation by AVP are rapid but sustained increases in channel $P_\text{o}$ followed by slower developing but substantial increases in the number of active channels in the apical membrane. As expected, cAMP and PKA are necessary for AVP-dependent increases in ENaC activity. Our finding that AVP increases ENaC activity is consistent with prior studies of AVP effects on the channel in immortalized amphibian distal nephron epithelial cells (30). Moreover, they agree with previous studies on isolated perfused rat collecting ducts showing that AVP stimulates apical-to-basolateral Na$^+$/H$^+$ transport in an amiloride-sensitive manner associated with decreases in resistance (and increases in conductance) and development of a lumen negative potential (11, 17, 20, 21, 36, 37, 42, 48). Amiloride blocks ENaC (18). Activating a luminal membrane Na$^+$/H$^+$ channel, such as ENaC, would be expected to decrease resistance (and increase conductance) as well as hyperpolarize the lumen. ENaC is thought to be the primary, possibly only, Na$^+$-selective channel in the apical membrane of collecting duct principal cells (5, 18, 23). Together, these findings demonstrate that ENaC is a physiologically important target of AVP in the mammalian collecting duct and evoke the possibility that stimulated ENaC contributes to the antidiuretic actions of AVP.

Our results showing that AVP increases ENaC activity in isolated tubules argue that the antidiuretic actions of this hormone do not fully account for its antinatriuretic effects. The
rationale for this is that the emergent properties of the kidney are critical to the antidiuretic actions of AVP involving development and maintenance of an axial corticomedullary osmotic gradient, which eventuates decreases in distal nephron urine flow (reviewed in Refs. 2, 16, 40). Decreases in urine flow in the collecting duct may accommodate enhanced Na$^+$ reabsorption at this site facilitating an antinatriuretic response. However, by taking the collecting duct out of the kidney, we removed it from any higher ordered architecture leaving only the possibility of primary actions. That we see an AVP effect on ENaC in the isolated tubule then strongly argues that this is a primary response of the channel to the hormone rather than a secondary tissue level response involving increased Na$^+$ reabsorption due to decreases in flow. Thus, our results are consistent with AVP exerting a primary antinatriuretic effect involving direct activation of ENaC in the distal nephron. This then leads to the position that this activation of ENaC may contribute to the antidiuretic response of AVP by supporting maintenance of the axial corticomedullary osmotic gradient. Restating this, our results are most consistent with the antinatriuretic effects of AVP contributing to the antidiuretic effects rather than visa versa.

Results from experiments investigating the interplay on ENaC activity of water-conserving and Na$^+$-conserving states strengthen this conclusion. Our results demonstrate that whole animal water restriction (for 18–24 h) and prolonged exposure (**30 min**) to exogenous AVP have similar effects on ENaC activity consistent with water restriction affecting ENaC via endogenous AVP. This is expected. Similar to that reported by the Schafer laboratory (11, 20), where they observed synergistic effects of aldosterone and AVP on Na$^+$ reabsorption in the isolated perfused rat collecting duct, we find that water and Na$^+$ restriction have additive effects increasing ENaC activity. Indeed, to reach maximal activity, ENaC had to come from mice deprived of both water and Na$^+$. This AVP and aldosterone convergence is consistent with the current understanding that AVP and cAMP/PKA signaling inhibit downregulation of ENaC (number) by phosphorylating the negative regulator Ned4 in parallel with aldosterone increasing ENaC activity by inhibiting Ned4 actions via stimulation of Sgk, which also impedes Ned4 binding to ENaC by phosphorylating this ubiquitin ligase (47). Importantly, we observed a significant increase in ENaC activity when mice were water restricted but maintained with high dietary [Na$^+$]; these animals are attempting to conserve water but not necessarily sodium. This AVP-dependent but aldosterone-independent increase in ENaC activity in collecting duct principal cells then likely contributes to regulation of systemic water balance rather than Na$^+$ balance supporting the idea that AVP-activated ENaC also contributes to renal water reabsorption. Possibly, it does so by contributing to or supporting the axial corticomedullary osmotic gradient.

The specific details of how activation of ENaC by AVP possibly contributes to water reabsorption though remain to be established. Understanding currently is confounded by findings in mice with compromised ENaC activity, suggesting that renal water handling may not be compromised in these animals (22, 23, 31). However, renal water handling in these animals, particularly under stressed conditions, has not been a focus of study and, thus, has not been tested thoroughly yet. Mice with tissue-specific deletion of the mineralocorticoid receptor in principal cells show increased water excretion when stressed with low salt (38). This is consistent with aldosterone-regulated ENaC playing some role in water reabsorption, which when compromised contributes to the inappropriate water loss observed in these knockout animals. Similarly, urinary concentrating defects are known to result from adrenal insufficiency (44) possibly emphasizing the importance of the additive effects AVP and aldosterone have on ENaC.

When comparing and contrasting the effects of water and sodium restriction, as well as exogenous AVP on increases in ENaC activity, we observed both similarities and differences. These are emphasized in the model presented in Fig. 5. As mentioned above, AVP and water restriction both increase ENaC $P_o$, but not as strongly as Na$^+$ restriction demonstrating that aldosterone has a quantitatively greater effect on this parameter of ENaC activity. This finding that AVP increases ENaC $P_o$ in murine principal cells within freshly isolated collecting ducts differs slightly from the actions of this hormone on ENaC in cultured amphibian distal nephron cells where the molecular mechanism is an increase only in $N$ and not $P_o$ (30). In collecting ducts, both water and salt restriction increase the frequency of ENaC being in the membrane with either alone capable of saturating frequency. Only Na$^+$ restriction though had trophic effects on the channel consistent with the established actions of aldosterone on ENaC protein levels (18, 51). We observed a subtle difference between the actions of water restriction and sodium restriction on ENaC membrane levels. The latter caused an increase in the frequency of observing ENaC as well as increasing the number of ENaC observed in any given membrane area, which we refer to as clustering. Water restriction, in contrast, increased only frequency and not clustering. Again, this observation in the mammalian collecting duct differs slightly from observations made in cultured amphibian epithelial cells where AVP increases the number of active ENaC in the apical membrane by increasing clustering (30). These subtle differences in mechanism of AVP action on ENaC in the mammalian and amphibian distal nephron may be real or rather the result of slight variations in experimental design and/or data collection and quantitation. Importantly, AVP significantly increases ENaC activity in both mammalian and amphibian distal nephron epithelium. We interpret our results as AVP, in addition to protecting ENaC numbers in the membrane by blocking the inhibitory actions of Ned4 (47), promoting trafficking of a primed vesicle pool containing ENaC to the luminal membrane. Similarly, aldosterone also likely does this but in addition, puts more ENaC into this primed vesicle pool increasing the clustering of ENaC in vesicles and thus, in the apical membrane as vesicles merge. The conclusion that AVP promotes movement of a primed vesicle pool toward the membrane is consistent with current understanding of the cellular actions of this hormone particularly with regards to AQP2 trafficking to the luminal membrane (8, 9, 46). Our contention is that some ENaC is in this AVP-sensitive vesicular pool. A contention that is consistent with the observations that AVP and cAMP promote ENaC trafficking to the luminal membrane and increase the number of ENaC in this membrane (6, 8, 13, 14, 46).
REFERENCES


