Amiloride-sensitive Na\textsuperscript{+} channels in pelvic uroepithelium involved in renal sensory receptor activation

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Our previous studies have demonstrated the presence of mechanosensitive nerve fibers in the renal pelvis. Graded increases in renal pelvic pressure result in graded increases in afferent renal nerve activity (ARNA), with the activation threshold being \textlessthan\text{5 mmHg} (24), a protein well below sensation of pain (50). Renal pelvic afferent nerves may play a role in the renal control of body water and sodium because activation of these afferent nerves results in a reflex-mediated increase in contralateral urinary sodium excretion (19-24). PGE\textsubscript{2} and substance P are important mediators of the signal elicited by increased renal pelvic pressure.

Our studies have shown that increasing renal pelvic pressure increases the release of PGE\textsubscript{2} into the renal pelvic effluent, which in turn increases the release of substance P. The released substance P activates substance P receptors in the renal pelvic area with a resultant increase in ARNA (19).

The majority of the afferent renal sensory nerves are located in the renal pelvic wall in the smooth muscle layer just beneath the transitional cells, with few fibers extending to the upper part of the calix (27, 49). Some nerve fibers appear to extend into the uroepithelial cell layer (49). Mechanical deformation of nerve endings produced by stretch is considered the primary mechanism of mechanoreceptor activation (3). Direct measurements of ionic current in the frog and mammalian muscle spindle preparation have shown that stretch is associated with an increase in cell membrane Na\textsuperscript{+} permeability, resulting in an inward flux of Na\textsuperscript{+} and depolarization (16, 34). Renal pelvic perfusion with NaCl at concentrations of 0.5 M and higher has been shown to increase basal ARNA (36) and enhance the ARNA response to increased renal pelvic pressure (24). In agreement with the numerous studies showing that inhibition of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase by ouabain lowers the activation threshold and enhances the responsiveness of carotid sinus and aortic baroreceptors (e.g., Refs. 3, 13), our previous studies showed that renal pelvic perfusion with ouabain decreases the threshold for activation of renal pelvic mechanosensitive nerves and enhances the ARNA response to increased renal pelvic pressure (24). The increased responsiveness of sensory nerves by ouabain is thought to be related to increased intracellular Na\textsuperscript{+} concentration (13).

In the kidney, the message for the \textalpha\textsubscript{1}, \textbeta\textsubscript{1}, and \textgamma-subunits of epithelial Na\textsuperscript{+} channels (ENaC) has been found in collecting duct cells (10, 46). In view of the modulatory role of Na\textsuperscript{+} in renal sensory receptor activation, it is of interest that Na\textsuperscript{+} channels and members of their genetic family are found in tissues that do more than absorb Na\textsuperscript{+} (14). ENaC show homology with genes involved in mechanical sensing and transduction (14, 35). Therefore, we examined whether the message for the \textalpha\textsubscript{1}, \textbeta\textsubscript{1}, and \textgamma-subunits of ENaC could be found in the renal pelvic wall. Because the uroepithelium in the pelvis is structurally similar to that in the ureter and urinary bladder, we also looked for the message of the three subunits of ENaC in these two organs.

These studies demonstrate the presence of the \textalpha\textsubscript{1}, \textbeta\textsubscript{1}, and \textgamma-subunits of ENaC in the pelvis, ureters, and bladder. Because the location of ENaC corresponds with the location of renal sensory nerves (27, 49), we examined whether amiloride, a known blocker of ENaC...
(18), would alter the ARNA response to various stimuli of the renal pelvic sensory nerves, such as increased renal pelvic pressure, substance P, and capsaicin. Because the results showed that amiloride reduced the ARNA response to increased renal pelvic pressure, we further explored whether the effect of amiloride was related to a decrease in Na+ influx by examining whether amiloride prevented the ouabain-mediated enhancement of the ARNA response to increased renal pelvic pressure.

METHODS

In Situ Hybridization

Adult Sprague-Dawley rats weighing 150–200 g were anesthetized with ketamine [10 mg/kg ip (Boehringer Ingelheim Animal Health, St. Joseph, MO)]. For preparation of paraffin-embedded sections, tissues were perfused via the left ventricle with 4% paraformaldehyde in PBS, pH 7.4 at 37°C. After removal, sliced kidneys, ureters, and urinary bladder were immersed in the fixative for 2 h at 4°C and embedded in paraffin. Tissues for frozen section were rapidly removed, and the kidneys were sliced to 2-mm thickness and snap frozen in liquid isopentane. Tissues were sectioned and mounted as previously described (30, 47).

The prehybridization, hybridization, and development steps were conducted according to the general methods described previously (48), as adopted by this laboratory (30, 47). Sections were treated with 1 µg/ml proteinase K at 37°C for 40 min and acetylated with 0.1 M triethanolamine and 0.25% acetic anhydride at room temperature.

The [35S]UTP-labeled riboprobe was added to the hybridization solution at a final concentration of 12,000 cpm/µl. Hybridization was conducted for 16–20 h at 50°C, and the slides were then washed at high stringency at 60°C for 30 min and treated with RNase A and RNase T1. The sections were coated with NTB-2 autoradiography emulsion (Eastman Kodak, Rochester, NY) and exposed for 4–13 wk at 4°C before being developed and counterstained with toluidine blue.

RNase Protection Assay

Sprague-Dawley rats weighing 250–300 g were anesthetized with methoxyflurane and decapitated, and small pieces (<0.25 cm2) of kidney cortex and the entire inner medulla, pelvis, ureters, and urinary bladder were rapidly removed and immersed in liquid nitrogen. Total RNA was isolated from tissues using the method of Chomczynski and Sacchi (8). After precipitation with isopropanol, the RNA was rinsed with 75% ethanol and resuspended in diethyl pyrocarbonate-treated water. RNA from the inner medulla was subjected to an additional step by centrifugation through RNeasy (Qiagen, Chatsworth, CA) to reduce contaminating materials that seem to be unique to this tissue. This technique permits recovery of >90% of starting RNA.

The probes used for the RNase protection assay (RPA) were derived from those previously described for α-, β-, and γ-ENaC and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (46). The α-rat ENaC (rENaC) construct was a 422-nt segment representing a portion of the intracellular COOH terminal, the first membrane-spanning domain, and a portion of the extracellular domain. The construct was subcloned into the pKS(−) vector (Stratagene, La Jolla, CA) using restriction sites EcoRI and PstI. The β-rENaC construct was a 249-nt segment representing a portion of the extracellular domain within the cysteine-rich region but not including the second membrane-spanning domain. The fragment was subcloned into the vector PCR-Script SK(+) (Stratagene) using restriction sites PstI and SacI. The γ-rENaC construct was a 675-nt segment representing slightly over 50% of the extracellular domain including the cysteine-rich region. This fragment was subcloned into the pCRII-TA vector (Invitrogen, Carlsbad, CA) using restriction sites XbaI and BstXI. The rat GAPDH construct was a 140-nt segment extending from the translation start site to the first StyI restriction site.

Antisense probes for the RPA were synthesized from the appropriate constructs using the BrightStar BIOTINiSCT nonisotopic in vitro transcription kit (Ambion, Austin, TX). The amount of biotin-labeled CTP was adjusted to give the highest possible specific activity. The lengths of the biotin-labeled, unprotected fragments were 480, 280, 750, and 220 nt for α-, β-, and γ-rENaC and GAPDH, respectively.

The hybridization of ~1 ng of each of these probes with 25 µg total RNA from each of the tissues was conducted using the RPA II RPA kit (Ambion). The products were subjected to electrophoresis through a 5% denaturing polyacrylamide–8 M urea gel buffered with Tris borate for 2.5 h at 250 V and transferred to a nylon membrane (BrightStar Plus, Ambion) using a semi-dry electrophorot (Fisher, Itasca, IL). The protected RNA fragments were detected using the BrightStar Biodec nonisotopic detection kit (Ambion) with minor modifications. The developed blots were exposed to Kodak BAR-5 film (Eastman Kodak, Rochester, NY) for 1 to 45 min depending on the intensity.

RT-PCR

Using RNA prepared from pelvis, renal inner medulla, and urinary bladder as described above, we conducted the reverse transcription as previously described (30). The PCR for α-ENaC was conducted in two steps using a nested strategy for increased sensitivity and specificity. The first round used the following primers to yield a product of 585 bp: forward primer, 5′-GACTGGAAGATCGGCTTCCA; reverse primer, 5′-CACCTTGCTTGATACCTTGAAGGG. These primers are designed to amplify a segment corresponding to a portion of the extracellular domain downstream from and not overlapping with the region used for the RPA and the in situ hybridization. About 15 ng of this material was subsequently subjected to another round using the following (nested) primers to yield a product of 353 bp: forward primer, 5′-TGGTGGTTCGTTCTC; reverse primer, 5′-GAGTGAAGTCTTGTCTA. The PCR reactions were conducted using 0.8 µM of the appropriate primers, 2 mM Mg2+, and 4 µl of the RT reaction product in a total volume of 50 µl. After an initial denaturation step (94°C for 5 min), the reaction underwent 35 cycles of denaturing (94°C for 1 min), annealing (63°C for 1 min), and extending (72°C for 1 min). An aliquot (4 µl) of this reaction was subjected to a second round of PCR under the same conditions. Aliquots (25 µl) of the reactions were subjected to electrophoresis on a 1.8% agarose gel and visualized with ethidium bromide.

In Vivo Study

The study was performed on male Sprague-Dawley rats weighing 233–424 g (mean weight 319 ± 4 g). Anesthesia was induced with pentobarbital sodium, 0.2 mmol/kg ip (Abbott Laboratories), and maintained with an intravenous infusion of pentobarbital sodium, 0.04 mmol·kg−1·h−1, in isotonic saline at 50 µl/min, into the femoral vein. Arterial pressure was recorded from a catheter in the femoral artery. The procedures for stimulating and recording ARNA have been developed using restriction sites R1781

ENAC INVOLVED IN RENAL PELVIC SENSORY ACTIVATION

R1781

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previously described in detail (19–24). In short, the left kidney was approached by a flank incision, a PE-10 catheter was placed in the right ureter for collection of urine, and a PE-60 catheter was placed in the left ureter with its tip in the renal pelvis. The left renal pelvis was perfused, via a PE-10 catheter placed inside the PE-60 catheter, throughout the experiment at 20 µl/min with vehicle or various renal perfusates administered in the different experimental protocols. ARNA was stimulated by increasing renal pelvic pressure or administering substance P or capsaicin (see below) into the renal pelvis via the PE-10 catheter. Renal pelvic pressure was increased by elevating the catheter above the level of the kidney. ARNA was recorded from the peripheral portion of the cut end of one renal nerve branch placed on a bipolar silver wire electrode. ARNA was integrated over 1-s intervals, the unit of measure being microvolts per second per second. Postmortem renal nerve activity, which was assessed by crushing the decentralized renal nerve bundle peripheral to the recording electrode, was subtracted from all values of renal nerve activity. ARNA was expressed in percentage of its baseline value during the control period (19–24).

Experimental Protocols

Approximately 1.5 h elapsed after the end of surgery and the start of the experiment to allow the rat to stabilize as evidenced by 30 min of steady-state urine collections and ARNA recordings.

Effects of amiloride and benzamil on the ARNA response to increased renal pelvic pressure. The experiment consisted of three parts separated by 20-min intervals. Each part consisted of a 10-min control, 5-min experimental, and 10-min recovery period. Renal pelvic pressure was increased during each experimental period. Renal pelvis was perfused with vehicle during the first part, 1 mM amiloride during the second (middle) part, and vehicle during the third part. The renal pelvic perfusates were switched immediately after each recovery period. Thus the renal pelvis was perfused with amiloride or benzamil for 30 min before renal pelvic pressure was increased during the second part of the experiment. In an additional five rats, a similar experimental protocol was performed, except benzamil at 0.001 and 0.01 mM benzamil (n = 10) during the second (middle) part, and vehicle during the third part. The renal pelvic perfusates were switched immediately after each recovery period. Thus the renal pelvis was perfused with amiloride or benzamil for 30 min before renal pelvic pressure was increased during the second part of the experiment. In an additional five rats, a similar experimental protocol was performed, except benzamil at 0.001 and 0.01 mM benzamil (n = 10) during the second (middle) part, and vehicle during the third part. The renal pelvic perfusates were switched immediately after each recovery period. Thus the renal pelvis was perfused with amiloride or benzamil for 30 min before renal pelvic pressure was increased during the second part of the experiment. In an additional five rats, a similar experimental protocol was performed, except benzamil at 0.001 and 0.01 mM benzamil (n = 10) during the second (middle) part, and vehicle during the third part. The renal pelvis was perfused with vehicle during the first part and 1 mM amiloride during the first part, 1 mM amiloride during the second part, and 1 mM amiloride plus 1.4 mM ouabain during the third part of the experiment. Thus renal pelvis was perfused with the various agents for 15 min before renal pelvic pressure was increased.

Effects of amiloride on the ARNA response to substance P. The experiment consisted of three parts separated by 20 min (n = 7). Each part consisted of two 5-min experimental periods, each bracketed by a 10-min control and recovery period. Substance P, 0.15 nmol, was administered into the renal pelvis during the first experimental period, and renal pelvic pressure was increased during the second experimental period. Renal pelvis was perfused with vehicle during the first part, 1 mM amiloride during the second (middle) part, and vehicle during the third part.

Effects of amiloride on the ARNA response to capsaicin. The experiment consisted of two parts separated by a 20-min interval (n = 7). Each part consisted of a 10-min control, 3-min experimental, and 10-min recovery period. Capsaicin at 0.04 nmol was administered during the two experimental periods. Renal pelvis was perfused with vehicle during the first part and 1 mM amiloride during the second part.

Drugs. All agents were from Sigma Chemical (St. Louis, MO), unless otherwise stated. Amiloride was dissolved in 0.15 mM NaCl. Benzamil was dissolved in DMSO and further diluted with 0.15 mM NaCl to a final DMSO concentration of 0.1%. Substance P and capsaicin were dissolved in 0.15 mM NaCl and further diluted in the various renal perfusates administered in the different experimental protocols.

Statistical Analysis

Systemic hemodynamics and renal excretion were measured and averaged over each period. The ARNA responses to the various stimuli were calculated as the area under the curve of time vs. ARNA, where ARNA was expressed as a percentage of its baseline value during the 10-min control period preceding each experimental period.

Friedman two-way ANOVA with multiple comparisons between groups, Kruskal-Wallis one-way ANOVA with multiple comparisons between treatments, Mann-Whitney test, and Wilcoxon matched-pairs signed-rank test were applied to test the significance between groups, two unrelated samples and two related samples, respectively (41). A significance level of 5% was chosen.

RESULTS

In Situ Hybridization

The results of in situ hybridization of kidney and pelvis for α-, β-, and γ-rENaC are shown in Fig. 1. The cortical collecting ducts show prominent hybridization with all three subunits, whereas there is no evident hybridization with inner medullary collecting ducts. There was also a prominent hybridization of β- and γ-rENaC, but not α-rENaC, along the pelvic uroepithelium. The intensity of the β- and γ-rENaC hybridization diminished substantially at the upper part of the pelvis where the uroepithelium juxtaposes with the cortex of the upper and lower poles. There was no hybridization with papillary epithelial cells.

Figure 2 shows sections of the urinary bladder hybridized with each of the rENaC probes. The transitional cells of both the bladder and the distal ureter hybridize with β- and γ-rENaC but not α-rENaC. The pattern of the signal within the uroepithelium was similar in all regions of the bladder. Figure 3 shows sections of the upper ureter demonstrating hybridization with β-, and γ-rENaC but not α-rENaC in the uroepithelium. Taken together, these in situ hybridizations demonstrate localization of β-, and γ-rENaC but not α-rENaC to the transitional cells of the renal pelvis, urinary bladder, and ureters.
RPA and RT-PCR

We estimated the relative amount of each of the subunit mRNAs by RPA in various regions of the kidney, ureters, and urinary bladder. As shown in Fig. 4, kidney cortex displayed abundant amounts of all three subunits, whereas inner medulla displayed smaller amounts of all three subunits. The ureter and urinary bladder showed abundant β- and γ-rENaC, but α-rENaC was either absent or too low to detect. In the pelvis, we could detect faint bands for all three subunits. Thus the RPA suggested a difference in the relative abundance of α-rENaC in the pelvis, ureters, and urinary bladder.

Having demonstrated β- and γ-rENaC mRNA throughout the uroepithelium by both in situ hybridization and RPA, we turned our attention to α-rENaC. We used RT-PCR because it is the most sensitive assay. The results of the strategy using a nested approach (for greater sensitivity and specificity) directed at a different region of the α-rENaC mRNA is shown in Fig. 5. As expected (46), α-rENaC was identified in inner medulla. α-rENaC was also detected in the urinary bladder and in the renal pelvis. The less intense bands in the latter two tissues compared with the inner medulla probably reflect a lower content of endogenous α-rENaC mRNA in the urinary bladder and renal pelvis.

In Vivo Study

Effects of amiloride and benzamil on ARNA response to increased renal pelvic pressure. We tested the idea that rENaC participates in the activation of the ARNA response to renal pelvic stress by perfusing the pelvis with inhibitors of rENaC. Increasing renal pelvic pressure 21 ± 6 mmHg results in reversible increases in ipsilateral ARNA (Fig. 6) and contralateral urinary sodium excretion (Table 1) before renal pelvic perfusion with 1 mM amiloride and 0.1 mM benzamil, respectively. Renal pelvic perfusion with either amiloride or benzamil did not alter basal ARNA. However, the ARNA response to increased renal pelvic pressure was reduced, in a reversible fashion, by either amiloride or benzamil (Fig. 6). The magnitude of reductions produced by amiloride, 53 ± 10%, and benzamil, 40 ± 10%, were not different. Administration of amiloride and benzamil also blocked the blocked the increases in contralateral urinary sodium excretion produced by increased renal pelvic pressure (Table 1).

In a separate group of rats, renal pelvic perfusion with benzamil at 0.001 and 0.01 mM failed to reduce the ARNA responses to increased renal pelvic pressure, the ARNA responses being 8,327 ± 136, 8,525 ± 1,541, and 7,790 ± 1,764 %·s (all P < 0.05) during renal pelvic
perfusion with vehicle, 0.001 mM benzamil, and 0.01 mM benzamil.

Increasing renal pelvic pressure did not affect mean arterial pressure. There was a gradual fall in mean arterial pressure throughout the experiment, from 116 $\pm$ 4 to 109 $\pm$ 2 mmHg ($P < 0.01$) in the group treated with amiloride and from 103 $\pm$ 2 to 98 $\pm$ 2 mmHg ($P < 0.05$) in the group treated with benzamil, 0.1 mM. The fall in mean arterial pressure was most likely not related to the administration of amiloride and benzamil since mean arterial pressure did not return to its control value when the renal pelvic perfusate was switched from amiloride or benzamil back to vehicle. Heart rate, 299 $\pm$ 14 and 314 $\pm$ 5 beats/min, remained unchanged throughout the experiment in the two groups.

Effects of amiloride on the ouabain-mediated enhancement of the ARNA response to increased renal pelvic pressure. The reduction of the ARNA response to increased renal pelvic pressure by amiloride and benzamil may have been the result of inhibition of a Na$^+$ channel. If such was the case, stretch would be expected to increase intracellular Na$^+$ concentration and cause depolarization (16, 34). Our previous studies have shown that ouabain, which increases intracellular Na$^+$ concentration and depolarizes the cell, enhances the ARNA response to renal pelvic wall stretch (24). We reasoned that if the ouabain-mediated enhancement of the ARNA response was the result of increased Na$^+$ influx through ENaC, pretreatment with amiloride should blunt the exaggerated ARNA response to pelvic wall stretch produced by ouabain. This hypothesis was examined in four protocols, each of which involved increasing renal pelvic pressure three times. The results are shown in Figs. 7–9. Increasing renal pelvic pressure 7.9 $\pm$ 0.1 mmHg during renal pelvic perfusion with vehicle produced similar increases in ARNA in the four groups (Figs. 7 and 9). Increasing renal pelvic pressure three times in the presence of vehicle resulted in reproducible increases in ipsilateral ARNA (group 1, Fig. 7A). In group 2, amiloride treatment reduced the ARNA responses to increased renal pelvic pressure compared with the ARNA response in the presence of vehicle (Fig. 7B). In the absence of amiloride (group 3), renal pelvic perfusion with ouabain resulted in a transient increase in basal ARNA (Fig. 8) that lasted 55 $\pm$ 9 s. Basal ARNA had returned toward control value before the start of the third experimental period. Ouabain enhanced the ARNA response to increased renal pelvic pressure in every rat ($P < 0.01$, Fig. 7C). In group 4, renal pelvic perfusion with amiloride reduced the ARNA response to increased renal pelvic pressure.

![Fig. 2. In situ hybridization of α (A and B), β (C and D), and γ (E and F)-rENaC with urinary bladder. Arrows show transitional epithelial cells lining the bladder lumen in bright-field (A, C, and E) and dark-field (B, D, and F) exposures. Ureter (u) shown as it traverses the muscular layers of the bladder wall.](http://ajpregu.physiology.org/DownloadedFrom/10.1152/ajpregu.00115.2017)
to a similar extent as in group 2 (Figs. 7 and 9). Adding ouabain to the amiloride-treated kidney resulted in a transient increase in basal ARNA that was less than that produced in the absence of amiloride in group 3 (P, 0.02, Fig. 8). Ouabain failed to enhance the ARNA response to increased renal pelvic pressure in the presence of amiloride in most rats (Fig. 9). However, in some rats, the ARNA response was greater in the presence of ouabain + amiloride than in the presence of vehicle. Further analysis of the data showed that the effect of amiloride on the ARNA response to increased renal pelvic pressure was varied (Fig. 9B). The effect of adding ouabain to the amiloride-treated kidney was inversely correlated to the magnitude of the amiloride-mediated blockade of the ARNA response to increased renal pelvic pressure (P < 0.001, Fig. 10). Thus, if amiloride reduced the ARNA response to increased renal pelvic pressure by more than 50%, addition of ouabain did not enhance the ARNA response. Conversely, if the amiloride-mediated inhibition was less than 50%, the addition of ouabain to the renal pelvic perfusate enhanced the ARNA response to increased renal pelvic pressure.

Mean arterial pressure, 123 ± 5, 123 ± 6, 124 ± 2, and 115 ± 2 mmHg, remained unchanged throughout the experiment in the four groups.

Effects of amiloride on the ARNA response to substance P. Increasing renal pelvic pressure increases renal pelvic release of substance P, and activation of renal pelvic substance P receptors contributes to the ARNA response to increased renal pelvic pressure (19,
23). We therefore examined whether amiloride reduced the ARNA response to substance P. The results are shown in Fig. 11. Before renal pelvic perfusion with amiloride, renal pelvic administration of substance P produced reversible increases in ipsilateral ARNA (Fig. 11) and contralateral urinary sodium excretion, from 0.77 ± 0.20 to 0.99 ± 0.28 µmol·min⁻¹·g⁻¹ (P < 0.05). Amiloride produced a reversible reduction of the ARNA response to substance P. Likewise, the natriuretic response to substance P was blocked by amiloride. In these same rats, amiloride inhibited (by 37 ± 12%) the increase in ARNA produced by increasing renal pelvic pressure 20 ± 1 mmHg. This effect was similar to that shown in Fig. 6. Mean arterial pressure, 110 ± 2 mmHg, and heart rate, 286 ± 45 beats/min, remained unchanged throughout the experiment.

Effects of amiloride on the ARNA response to capsaicin. To assess whether the amiloride-mediated reduction of the ARNA responses to increased renal pelvic pressure and substance P was due to a nonspecific desensitization of the renal sensory nerves, we examined whether amiloride altered the ARNA responses to capsaicin. Capsaicin depolarizes sensory neurons by increasing the conductance of nonselective cation channels with a preference for Ca²⁺ (5). Renal pelvic administration of capsaicin resulted in increases in ipsilateral ARNA (Fig. 12) and contralateral urinary sodium excretion, from 1.11 ± 0.20 to 1.34 ± 0.25 µmol·min⁻¹·g⁻¹ (P < 0.05). The ipsilateral ARNA and contralateral natriuretic responses to capsaicin were not affected by amiloride. Mean arterial pressure, 115 ± 6 mmHg, and heart rate, 325 ± 8 beats/min, were unchanged throughout the experiment.

DISCUSSION

The results of these experiments show the presence of the mRNA for α-, β-, and γ-subunits of ENaC in the uroepithelium. The functional studies show that the ARNA response to increased renal pelvic pressure is markedly blunted by renal pelvic perfusion with either amiloride or benzamil, known blockers of ENaC (18). Furthermore, amiloride prevents the enhancement of

Fig. 5. Representative RT-PCR designed to identify α-ENaC mRNA. A: initial PCR using primers designed to amplify 585 bp of α-ENaC. B: nested PCR using material from the initial reaction designed to amplify 353 bp of α-ENaC mRNA. Each reaction product is the predicted length.

Fig. 6. Afferent renal nerve activity (ARNA) responses to increased renal pelvic pressure in presence of renal pelvic perfusion with vehicle, 1 mM amiloride, and vehicle (A) or vehicle, 0.1 mM benzamil, and vehicle (B); n = 10 (both groups). AUC, area under the curve of ARNA vs. time. *P < 0.05, **P < 0.01.
the ARNA response to increased renal pelvic pressure produced by inhibition of Na\(^+\)-K\(^+\)-ATPase with ouabain. These functional data together with the morphological data suggest that the rENaC complex in the renal pelvic uroepithelium participates in the activation of renal pelvic mechanosensitive neurons.

**ENaC Proteins and Mechanosensation**

We postulated that the major role of ENaC in the uroepithelium may not be related to Na\(^+\) reabsorption. In contrast to amphibian bladders, which can absorb large amounts of Na\(^+\) in response to aldosterone (37), mammalian urinary bladders transport little Na\(^+\) under normal conditions (28). Although little is known about the Na\(^+\) transport capabilities of the renal pelvic uroepithelium, the similar epithelial structure of the bladder and pelvic epithelium would suggest that little transepithelial Na\(^+\) transport occurs in mammalian renal pelvic uroepithelium. We hypothesize that the ENaCs in the uroepithelium are involved in mechanosensation. Studies in rabbit urinary bladder show that stretch produces a transient Na\(^+\) current, part of which is sensitive to amiloride (28). Furthermore, sequence analysis of rENaC has shown that the three subunits are highly homologous to mechanosensitive genes found in Caenorhabditis elegans (14). Mutations in MEC-4 or MEC-10 render the worm unable to recoil to touch (15). Several homologous proteins have been cloned from mammalian neural tissue. Recently, a combination of two related but separate such molecules has produced amiloride-sensitive Na\(^+\) currents when expressed in oocytes (2). Stretch-activated channels may form a functional subset of amiloride-sensitive channels. Such amiloride-sensitive channels include stretch-activated channels in *Xenopus* oocyte and cochlear hair cells that are characterized by higher conductance and lower affinity for amiloride than ENaC in lung, colon, and renal tubules (14, 35, 39). Although there is evidence for the stretch-activated channels being cation nonspecific, recent studies in tetrodotoxin-treated spider slit sense organs demonstrated that the current elicited by mechanical stimulation of the slits was dependent on extracellular Na\(^+\) (17).

In the kidney, the majority of the renal sensory neurons are located in the pelvic wall smooth muscle layer, with some fibers reaching into the uroepithelium. The greatest density of fibers in the pelvis occurs toward the ureters, with diminishing number of fibers

### Table 1. Effects of amiloride and benzamil on the contralateral natriuretic responses to increased renal pelvic pressure

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>↑ RPP</th>
<th>Recovery</th>
</tr>
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<tbody>
<tr>
<td>Vehicle</td>
<td>0.54 ± 0.20</td>
<td>0.75 ± 0.29*</td>
<td>0.63 ± 0.23</td>
</tr>
<tr>
<td>Amiloride</td>
<td>0.95 ± 0.28</td>
<td>1.09 ± 0.32</td>
<td>1.12 ± 0.28</td>
</tr>
<tr>
<td>Vehide</td>
<td>1.17 ± 0.22</td>
<td>1.49 ± 0.31†</td>
<td>1.3 ± 0.28</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.99 ± 0.35</td>
<td>1.32 ± 0.49†</td>
<td>1.00 ± 0.33</td>
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<tr>
<td>Benzamil</td>
<td>1.18 ± 0.34</td>
<td>1.46 ± 0.42</td>
<td>1.53 ± 0.39</td>
</tr>
<tr>
<td>Vehide</td>
<td>1.71 ± 0.41</td>
<td>2.00 ± 0.45†</td>
<td>1.80 ± 0.39</td>
</tr>
</tbody>
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Values are means ± SE. ↑ RPP, increased renal pelvic pressure; UNaV, urinary sodium excretion. *P < 0.01, †P < 0.02, ‡P < 0.05 vs. average of control and recovery periods.

Fig. 7. ARNA responses to increased renal pelvic pressure in presence of vehicle (n = 6) (A); vehicle, 1 mM amiloride, and amiloride (n = 6) (B); and vehicle, vehicle, and 1.4 mM ouabain (n = 8) (C). *P < 0.05, **P < 0.01.
in the upper part of the pelvis (27, 49). The spatial arrangement of rENaC within the uroepithelial cells in close vicinity of renal sensory nerves adds further circumstantial evidence to support the idea that rENaC molecules in uroepithelial cells might participate in mechanosensitive transduction to the afferent renal nerves.

Stoichiometry of the Subunits of ENaC

It is generally agreed that all three subunits of ENaC are required to form a functioning Na\(^+\) channel (4, 31). All three subunits are present in cortical collecting ducts, colon, and airway epithelial cells (1, 10, 30, 45), tissues where transepithelial Na\(^+\) transport is a major function of the epithelium. However, in the uroepithelium of the rat pelvis, ureters, and urinary bladder, the expression of \(\beta\)- and \(\gamma\)-rENaC mRNA is substantially greater than \(\alpha\)-rENaC, as shown in the present study. Our studies suggest that \(\alpha\)-rENaC mRNA might be relatively more abundant in the renal pelvis than in the bladder or ureters. However, the data from the RT-PCR suggest that it is not very abundant in either pelvis or bladder. This relative expression is different from that recently reported by Smith et al. (42) in rat urinary bladder. They showed a greater abundance of \(\alpha\)- and \(\beta\)-rENaC than \(\gamma\)-rENaC mRNA. They also provided immunocytochemical evidence for apical membrane localization of \(\alpha\)-rENaC in rat urinary bladder. The reason(s) for the difference in relative mRNA abundance in the bladder between the two studies are currently not known. One may speculate that different experimental conditions may underlie the different relative expression in the bladder in the two studies. For example, it is well known that the expression of mRNA ENaC subunits can be modulated by diet NaCl concentration and aldosterone (1, 44).

A possible explanation for the relatively low abundance of \(\alpha\)-rENaC in uroepithelium in the present study is that the stoichiometry of the functional ENaC unit in the uroepithelium is different from the Na\(^+\)...
channel in the collecting duct. There is substantial evidence for differential expressions of the three sub-units of ENaC in various tissues. In the respiratory tract, considerable variations in the expression of the three subunits of rENaC has been demonstrated within the same species (11). In the colon, only the α-subunit is expressed in the absence of aldosterone stimulation (1) and in the nodose ganglia, only β- and γ-subunits of rENaC were detected (9). Recent studies (32) indicate that variation in the composition of the three subunits results in channels of different functional properties with different amiloride affinity. These studies together with the present findings may indicate that the functional properties of the Na\(^{+}\) channels expressed in the uroepithelium differ from the ENaC expressed in collecting duct cells.

**Activation of Renal Sensory Receptors:**

Sensitivity of Amiloride

Our previous studies have suggested a physiological role for the renal pelvic mechanosensitive neurons in the renal control of water and sodium (21, 24). The present study shows that the increase in ARNA produced by increasing renal pelvic pressure was reduced by renal pelvic perfusion with amiloride or benzamil. The concentration of amiloride and benzamil required to inhibit the increase in ARNA was larger than that needed to inhibit ENaC activity in the collecting ducts and other tissue (12, 38) but was in the range of that required to block mechanosensitive channels in mouse hair cell and frog oocyte preparations (14), suggesting that the rENaC complex in the uroepithelium may be part of mechanosensitive channels. The higher concentration required in the present study may also be explained by the stoichiometry of rENaC not being the...
same as in the collecting duct, colon, and lung. Furthermore, the accessibility of amiloride to the ENaC protein complex in the uroepithelium may be limited. It is well established that the bladder uroepithelium is among the least permeable of all mammalian epithelia (e.g., Ref. 33). In this context, it is of interest that the majority of the Na⁺ current across the bladder epithelium was insensitive to conventional concentrations of amiloride, even after stretching (28). Moreover, as discussed below, our data do not exclude the possibility that the inhibitory effect of amiloride and benzamil on the ARNA response to the activation of renal sensory neurons also may have been related to an effect on theafferent nerve endings per se.

It may be argued that the high concentrations of amiloride and benzamil required to reduce the ARNA response to the activation of renal pelvic mechanosensitive neurons may indicate a blockade of Na⁺-channels other than ENaCs. However, the morphological evidence for α-, β-, and γ-ENaC in the renal pelvic uroepithelium together with the functional evidence for an inhibitory effect of amiloride and benzamil would imply that the rather large concentrations needed to inhibit the ARNA response to pelvic wall stretch may be explained by a different ENaC stoichiometry of the subunits in this tissue and/or the relative impermeability of the uroepithelium.

Amiloride-Mediated Blockade of Renal Sensory Receptor Activation: Possible Mechanisms

We hypothesized that if stretching the pelvic wall involves activation of a Na⁺ channel, the resultant increase in intracellular Na⁺ concentration would be an important factor in the activation of renal sensory neurons. It is well known that ouabain, an agent that increases intracellular Na⁺ concentration by virtue of its inhibition of Na⁺-K⁺-ATPase, increases the responsiveness of various sensory neurons, including the aortic and carotid baroreceptors (13) and renal pelvic mechanosensitive neurons (24). Our present findings showing that pretreating the renal pelvis with amiloride can prevent the ouabain-mediated enhancement of the ARNA response to increased renal pelvic pressure further support our hypothesis. The failure of amiloride to prevent the ouabain-mediated enhancement of the ARNA response in some rats was most likely explained by the effect of ouabain being inversely related to the magnitude of the amiloride-mediated blockade of the ARNA response to increased renal pelvic pressure. These findings suggest that if amiloride prevented Na⁺ influx, ouabain failed to enhance the ARNA response. On the other hand, if amiloride only partially blocked Na⁺ influx, ouabain would enhance the ARNA response. The differential effect of amiloride on the ouabain-mediated enhancement of the ARNA response may suggest the presence of stretch-activated Na⁺ channels that are amiloride sensitive and some that are not.

Our current findings suggest that rENaC may not only be involved in the renal sensory receptor activation by pelvic wall stretch. Renal pelvic administration of amiloride also reduced the ARNA responses to substance P and ouabain at unchanged renal pelvic pressure. Substance P plays an important role in the activation of renal sensory nerves. Renal pelvic sensory neurons contain substance P (27, 49), and substance P is released into the renal pelvic effluent in response to increased renal pelvic pressure and bradykinin (19, 20). Furthermore, renal pelvic perfusion with a substance P receptor antagonist reduced the ARNA response to renal sensory receptor activation (20, 23). Although substance P receptors have been localized to the renal pelvic area by autoradiography (7), the cellular localization of substance P receptors in the pelvis is currently unknown. The mechanisms underlying cell membrane depolarization by substance P depend on the particular cell type. However, most studies show that substance P depolarizes the cell membrane by increasing Na⁺ and Ca²⁺ influx, with a preference for Na⁺ in some cells, and decreasing K⁺ efflux (25, 40, 43). With this information as a background, we speculate that substance P may increase ARNA, at least in part, by activating amiloride-sensitive Na⁺ channels in the renal pelvic epithelium.

The sequence of events leading to cell membrane depolarization and an increase in ARNA following increased renal pelvic pressure is not currently known. Whether the increase in ENaC conductance following increased renal pelvic pressure is related to the stretching of the renal pelvic wall per se and/or the pelvic pressure-induced release of substance P (19) cannot be deduced from the current data. Also, our data do not exclude the possibility that the amiloride-blockable Na⁺ channels involved in the activation of renal pelvic sensory neurons are located on both neural and nonneural cells in the pelvis. Studies of amiloride-sensitive Na⁺ channels in the tongue have shown the presence of ENaC in both taste cells and adjacent nontaste cells (26). The authors suggested that depolarization of taste cells following salt application to the tongue is related to increased intracellular Ca²⁺ concentration and release of neurotransmitters. The presence of ENaC in adjacent nontaste cells could augment the depolarization of the taste cells (26). Interestingly, taste buds are innervated by substance P-containing neurons (29). We hypothesize that similar mechanisms may be involved in the activation of renal pelvic mechanosensitive neurons, i.e., increases in intracellular Na⁺ concentration in uroepithelial cells may facilitate depolarization of adjacent sensory nerve endings during pelvic wall stretch.

It is important to note that the reduction of the ARNA response by amiloride was not due to a general desensitization of the sensory nerves because amiloride was without effect on the ARNA response to capsaicin. Capsaicin depolarizes sensory nerves by activating a receptor that is exclusively expressed in small-diameter neurons. The capsaicin receptor was recently cloned and found to be homologous to store-operated Ca²⁺ channels (5).

In summary, the present study shows the presence of mRNA of the α-, β-, and γ-subunits of rENaC in the
renal pelvic uroepithelium. Renal pelvic administration of amiloride reduced the ARNA response to increased renal pelvic pressure. These findings taken together with the data showing that amiloride prevented the ouabain-mediated enhancement of the ARNA response to increased renal pelvic wall stretch suggest that rENaCs are a component of the mechanosensitive neurons in the renal pelvic wall. The fact that amiloride inhibited the response to substance P as well as mechanical stretch explains the possibility of complex interactions between the uroepithelium and the underlying afferent nerves.

Perspectives

From a teleological point of view, it would be logical for a mechanism within the uroepithelial cell to be responsive to stretch and to transfer this mechanical stimulus to the afferent nerves. Although our findings suggest that amiloride reduces the stretch-induced ARNA by inhibiting Na$^+$ influx into uroepithelial cells, we point out that other possibilities are possible. The fact that amiloride blocks this process. An important role for substance P as a mediator of salt perception is suggested by the localization of substance P receptors to the basolateral membrane of the taste cells (6). Thus one may speculate that the intake and excretion of sodium may be controlled, at least in part, by similar mechanisms.

This work was supported by grants from the Department of Veterans Affairs; The National Institutes of Health O’Brien Kidney Disease Center Grant DK-52617 and Heart, Lung, and Blood Institute, Specialized Center of Research Grant HL-55006; American Heart Association, Iowa Affiliate, Grants-In-Aid; and the University of Iowa Diabetes and Endocrinology Research Center.

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Received 17 April 1998; accepted in final form 13 August 1998.

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