Expression and localization of epithelial sodium channel in mammalian urinary bladder

PETER R. SMITH,1 SCOTT A. MACKLER,2 PHILIP C. WEISER,2 DAVID R. BROOKER,2 YOON J. AHN,2 BRIAN J. HARTE,2 KATHLEEN A. MCNULTY,2 AND THOMAS R. KLEYMAN2,3

1Department of Physiology, Allegheny University of the Health Sciences, Philadelphia 19129; and Departments of Medicine and Physiology, University of Pennsylvania and 2Department of Veterans Affairs Medical Center, Philadelphia, Pennsylvania 19104

The mammalian urinary bladder is a transient reservoir for water and various solutes filtered and excreted by the kidneys. The epithelium of the urinary bladder serves as a barrier to retain these solutes and water. In addition, the bladder epithelium can modify the urinary contents by reabsorbing or secreting specific solutes. The urinary bladder epithelium has been shown to undergo dramatic changes in architecture in response to changes in bladder volume. In the contracted bladder, cells lining the bladder lumen are typically goblet shaped and are characterized by a folded apical membrane and a prominent population of subapical vesicles (22, 25, 34). During filling, the bladder epithelium accommodates the volume increase by stretching the cells so that the luminal cells become more cuboidal in shape with a smooth apical membrane and by the insertion of subapical vesicles into the apical membrane (25, 34). Ultrastructural analysis has demonstrated that, during stretching, the number of subapical vesicles decreases with an increase in the luminal membrane (34). Bladder stretching results in an ~70% increase in apical surface area because of the addition of new membrane from this subapical pool, as measured by an increase in membrane capacitance (25).

Electrophysiological studies have demonstrated that epithelial Na⁺ channels are present in the apical plasma membrane of the mammalian urinary bladder, where they function in transepithelial Na⁺ reabsorption. These Na⁺-selective channels mediate the movement of Na⁺ out of the urinary space (2, 17, 39, 42) and are selectively inhibited by submicromolar concentrations of the diuretic amiloride (21). Both the hormone aldosterone and stretch of the bladder in response to changes in urinary volume have been shown to increase the number of functional Na⁺ channels in the apical membrane (13, 25, 26). Maximal Na⁺ transport rates across rabbit urinary bladder have been estimated to be as high as ~80 μmol/h (27), suggesting that Na⁺ transport across the urinary bladder may contribute to reducing Na⁺ concentration in the final urine in Na⁺-retaining states.

The epithelial Na⁺ channel (ENaC) recently has been cloned from colon, kidney, and lung and consists of three homologous subunits (α-ENaC, β-ENaC, and γ-ENaC) (2, 6). Significant amino acid sequence similarities across species suggest that ENaCs belong to a common gene family and are structurally related to putative mechanosensitive ion channels found in the nematode Caeorhabditis elegans (9), a Phe-Met-Arg-Phe-NH₂-activated Na⁺ channel cloned from the snail Helix aspersa (29), and amiloride-sensitive Na⁺ channels expressed in brain (16, 37, 45). To date, limited information is available regarding the biochemical and molecular characteristics of ENaCs expressed in the mammalian urinary bladder. In this report, we demonstrate by a combination of Northern blot analysis, in situ hybridization, and immunocytochemistry that ENaCs are expressed in the epithelium of the mammalian urinary bladder.

MATERIALS AND METHODS

Tissue preparation. Normal Sprague-Dawley rats were kept on a standard laboratory diet and had free access to fresh water. For in situ hybridization and Northern blot analysis, animals were euthanized by deep anesthesia with 100% CO₂. Urinary bladders were punctured with a hypodermic needle, and the urine was removed to collapse the bladder. Urinary bladders were subsequently removed, cut
into small pieces, and placed in ice-cold phosphate buffered saline (PBS). For immunocytochemistry, animals were killed, and bladders were rapidly excised. Bladders were subsequently rinsed in ice-cold PBS, cut into small pieces, and placed in ice-cold PBS before embedding for frozen sectioning.

Antibodies. An anti-rENaC antibody was generated by Lofstrand Laboratories (Gaithersburg, MD), using a synthetic peptide corresponding to residues 44–57 (GLGKDKEEQGLG) within the NH2-terminal intracellular domain of the a-subunit of rENaC (5). The peptide was conjugated to bovine serum albumin through a COOH-terminal cysteine residue before being used as an immunogen. Antibodies were purified, using a protein A-agarose column (Pierce, Rockford, IL), following the manufacturer’s protocol. An anti-Na+-K+-adenosinetriphosphatase (anti-Na+-K+-ATPase) antisem was a generous gift of Dr. Stephen Ernst (Univ. of Michigan) and has previously been described (10, 41).

In situ hybridization. Small pieces of urinary bladder were fixed in 3% paraformaldehyde prepared in ethyl pyrocatechate-treated PBS containing 5% sucrose. Samples were subsequently cryoprotected by infiltration in PBS containing increasing concentrations of sucrose, with the final sucrose concentration being 20%, placed in gelatin capsules (Poly-science, Warrington, PA), and snap frozen in liquid nitrogen. Samples were stored at –80°C until use. Five-micrometer-thick sections were sectioned and collected on Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA).

cDNAs encoding partial-length clones of mouse a-, b-, and g-ENaC subunits (390 bp, 207 bp, and 244 bp, respectively) were utilized (3). Each cDNA was linearized, and 2 µg were used as a template for the synthesis of antisense or sense complementary RNAs (cRNAs) [4 µl transcription buffer (Promega, Madison, WI), 0.5 µl 100 mM dithiothreitol, 8 µl of a nucleotide triphosphate mix (including digoxigenin-UTP), 1 µl ribosomal RNasin (40 units/µl), and the appropriate RNA polymerase (20 units) in a total volume of 20 µl at 37°C for 2–3 h]. The length and amount of each cRNA were verified, after phenol-chloroform extraction and ethanol precipitation, by ethidium bromide staining in a formaldehyde-agarose gel. Digoxigenin-labeled cRNAs were detected in situ with the use of an anti-digoxigenin antibody conjugated to alkaline phosphatase (8). Briefly, each cRNA probe [–2 ng/µl, in a hybridization solution containing 1× SSC (0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 5× Denhardt’s solution (0.1% polyvinylpyrrolidone, 0.1% Ficoll, and 0.1% bovine serum albumin), salmon DNA (500 µg/ml), yeast tRNA (250 µg/ml), and 50% formaldehyde] was allowed to hybridize overnight at 50°C to tissue sections, followed by washing twice with 2× SSC at room temperature for 5 min and then once with STE at room temperature for 5 min. Sections were subsequently treated with ribonuclease A at 37°C for 30 min to remove unhybridized probe and washed at high stringency with 2× SSC, 50% formamide at 50°C for 5 min, 1× SSC at room temperature for 5 min, and 0.5× SSC at room temperature for 5 min. Sections were then blocked with 2% goat serum for 30 min, and bound digoxigenin was detected by incubation with alkaline phosphatase-conjugated anti-digoxigenin antibodies (Boehringer Mannheim, Indianapolis, IN) at room temperature for 1 h, followed by color development using nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt as the substrate. Sense cRNAs were used to evaluate the specificity of probe hybridization.

Northern blot analysis. Total RNA was isolated from rat urinary bladder, using the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (7), and size fractionated in a 1.2% agarose-formaldehyde gel. The RNA was transferred onto a nylon membrane and probed with 32P-random prime labeled ~1.5-kb cDNA inserts of each ENaC subunit (15). The blots were washed at a high stringency (final wash, 0.1× SSC at 65°C) and exposed to a phosphor screen for imaging (Molecular Dynamics, Sunnyvale, CA).

Immunofluorescence microscopy. Urinary bladder samples were placed unfixed in gelatin capsules containing tissue-freezing compound (Triangle Biomedical Services, Durham, NC), frozen by submersion in liquid nitrogen, and stored at –80°C until use. Five-micrometer-thick sections were cut, using an IEC Minotome cytotype, and collected on either Superfrost Plus or gelatin-subbed glass slides. Before processing for immunofluorescence microscopy, sections were fixed for 1 min in ice-cold methanol and then allowed to air dry. Sections were rehydrated in PBS and then incubated for 1 h in PBS containing 10% normal goat serum (NGS) (Sigma, St. Louis, MO). Sections were incubated overnight at 4°C in either rabbit polyclonal anti-a- or b-ENaC antibody (1:200 dilution; 6.5 µg/ml final concentration) or rabbit polyclonal anti-Na+-K+-ATPase antisem (1:500 dilution) in PBS containing 1% NGS. After incubation in primary antibodies, sections were sequentially washed in PBS (twice for 5 min), phosphate-buffered NaCl containing 1% bovine serum albumin (three times for 5 min; twice for 30 min), and PBS (twice for 5 min). Sections were then incubated in a 1:200 dilution of Texas red-conjugated goat anti-rabbit immunoglobulin G (Cappel/Organon Teknika, Durham, NC) for 1 h at room temperature. Control consisted of preincubation of primary antibody with excess free peptide (15 µg/ml final concentration). After six washes in PBS, sections were mounted in glycerol-PBS (9:1 vol/vol) containing 0.1% phenylenediamine (Sigma), covered with glass coverslips, and sealed with nail polish. Sections were photographed on a Nikon Optiphot II microscope equipped for epifluorescence and differential interference microscopy.

RESULTS

Expression of ENaC subunit mRNAs in rat urinary bladder epithelium. The expression of ENaC subunit mRNA in the rat urinary bladder (Fig. 1) derived from animals maintained on a standard feed was examined by Northern blot analysis. Radiolabeled a-, b-, and g-ENaC probes hybridized to mRNAs of ~3.7, 2.6, and 3.2 kb in size, respectively, in agreement with the reported size in rENaC mRNAs in colon and kidney (Fig. 1) (6). In addition, slot blot Northern analyses were performed to examine the abundance of a-, b-, and g-ENaC mRNAs in rat urinary bladder, relative to b-actin expression (Table 1). Similar levels of expression of a- and b-ENaC mRNAs were observed. Interestingly, levels of expression of g-ENaC mRNA were ~5- to 10-fold lower than a- and b-ENaC mRNAs.

The expression and distribution of rENaC mRNAs were further examined by in situ hybridization. The bladder epithelium was found to vary from ~30 to 80 µm in height, depending on the extent of bladder contraction during preparation of the tissue for in situ hybridization. Signals for a- and b-ENaC mRNAs were detected in the urinary bladder epithelium of animals maintained on standard rat chow (Fig. 2, A and
No significant signal was obtained in parallel sections hybridized with control sense cRNA probes, demonstrating the specificity of the hybridization with the ENaC probes (Fig. 2, B and D). A weak specific signal for γ-rENaC mRNA was also detected in rat urinary bladder epithelia (Fig. 2, E and F).

**Immunolocalization of α-ENaC in the urinary bladder.** To further demonstrate expression of ENaC in the urinary bladder, the distribution of α-ENaC was examined by indirect immunofluorescence microscopy (Fig. 3), using an antibody generated against a peptide corresponding to amino acids 44–57 within the NH₂ terminus of α-rENaC. Immunostaining for α-rENaC was predominantly localized to the apical membranes of cells lining the bladder lumen (Fig. 3). In addition, a diffuse subapical intracellular staining was observed (Fig. 3). Immunostaining was specific, as preincubation of the antibody with excess free immunogenic peptide blocked antibody binding to the epithelial cells (Fig. 3).

**Immunolocalization of Na⁺-K⁺-ATPase in the rat urinary bladder.** Na⁺-K⁺-ATPase is expressed in the basolateral plasma membrane of most epithelial cells (18, 20). An anti-Na⁺-K⁺-ATPase α-subunit antibody was used to localize this transporter in the rat urinary bladder to provide a better definition of the architecture of this epithelium and to help delineate the basolateral plasma membrane of the urinary bladder epithelium. As shown in Fig. 4, Na⁺-K⁺-ATPase localized to the basolateral membrane of the bladder epithelial cells.

**Table 1.** Expression of rENaC subunits in rat urinary bladder, relative to β-actin expression

<table>
<thead>
<tr>
<th>Subunits</th>
<th>Relative Expression</th>
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<tbody>
<tr>
<td>α-rENaC</td>
<td>1.98 ± 0.99*</td>
</tr>
<tr>
<td>β-rENaC</td>
<td>1.74 ± 0.37†</td>
</tr>
<tr>
<td>γ-rENaC</td>
<td>0.23 ± 0.01</td>
</tr>
</tbody>
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Values are means ± SE; n = 3. rENaC, rat epithelial Na⁺ channel.

*P < 0.08, alpha vs. gamma; †P < 0.01, beta vs. gamma.
DISCUSSION

Several ENaCs have recently been described. These include a family of structurally related two-membrane-spanning-domain polypeptides (α-, β-, and γ-ENaC), which assemble into an oligomeric Na\(^{+}\) channel. A guanosine 3',5'-cyclic monophosphate-regulated amiloride-sensitive cation channel is expressed in renal and airway epithelia and is structurally related to a cation channel expressed in rod outer segment (19). An apical membrane protein cloned from a Xenopus renal epithelial cell line, termed Apx, is an amiloride-sensitive cation channel (36) or a cation channel regulator (43). Although previous electrophysiological studies have indicated that amiloride-sensitive Na\(^{+}\)-selective ion channels (13, 24, 26–28, 35) are functionally expressed in the apical plasma membrane of the urinary bladder epithelium, their molecular identity was unknown.

We provide data in this paper that the recently cloned ENaCs are expressed in the rat urinary bladder. In situ hybridization and Northern blot analyses indicate that α-, β-, and γ-ENaC are expressed in the rat urinary bladder. Quantitation of the levels of α-, β-, and γ-ENaC mRNA expression in the rat urinary bladder, relative to β-actin mRNA expression, suggests that, although comparable levels of α- and β-ENaC subunits are expressed in the urinary bladder of rats maintained on standard chow, the level of γ-ENaC mRNA expression is 5- to 10-fold lower than either α- or β-ENaC. This resembles the pattern of ENaC mRNA expression in human airway epithelia (4) and the M-1 cortical collecting duct cell line (23), where γ-ENaC subunit mRNA expression is significantly lower than the expression of α- and β-ENaC mRNAs. Differential expression of the three ENaC subunits has been observed in several tissues, including lung, liver, skin, and colon (1, 4, 14, 30, 32, 33, 38, 40). In light of ENaC being viewed as a heteromeric channel being composed of three subunits (6), the functional consequences of different levels of ENaC subunit mRNA expression within the same tissue are presently not understood.

Amiloride-sensitive Na\(^{+}\) transport across the mammalian urinary bladder is stimulated by the mineralocorticoid hormone aldosterone or by feeding animals an Na\(^{+}\)-restricted diet (13, 27). Several candidate mecha-
nisms for the regulation of Na\(^+\) channel by aldosterone have been described, and regulatory mechanisms may differ among different tissues or cell types. These mechanisms include posttranslational modification of a channel subunit or an associated regulatory protein (i.e., G proteins or membrane cytoskeleton) and increases in ENaC mRNA and protein expression (reviewed in Refs. 2 and 31). With respect to the latter, aldosterone (or low dietary Na\(^+\)) increases the level of expression of \(\beta\)- and \(\gamma\)-ENaC mRNA in the rat colon (1, 30). However, in kidney and primary cultures of cortical collecting duct and of inner medullary collecting duct cells, responses to aldosterone (or low dietary Na\(^+\)) have included no change in ENaC subunit mRNA expression (38) or a small increase in \(\alpha\)-ENaC mRNA (1, 44) or in \(\gamma\)-ENaC mRNA expression (11). In light of the foregoing discussion, our observations of low levels of \(\gamma\)-ENaC mRNA expression in the urinary bladders from animals fed a standard diet raise the possibility that the aldosterone-induced increase in Na\(^+\) transport across the urinary bladder may be, at least in part, mediated by increases in \(\gamma\)-ENaC mRNA and protein levels.

To further corroborate expression of ENaC in the urinary bladder, we examined the distribution of the \(\alpha\)-subunit of ENaC by immunofluorescence microscopy. \(\alpha\)-ENaC was localized primarily to the apical membranes of the cells lining the lumen of the rat urinary bladder. This localization of ENaC to the apical membrane domain is in agreement with recent immunocytochemical studies demonstrating that ENaCs are localized to the apical membrane in the kidney, distal colon, lung, sweat ducts, and parotid salivary glands (12, 38). In addition, a diffuse subapical cytoplasmic staining of the luminal cells was observed, suggesting that ENaC may also be localized to a population of intracellular vesicles. Lewis and de Moura (25, 26) have demonstrated that during urinary bladder distension, there is a change in membrane capacitance, presumably resulting from an increase in apical membrane area induced by vesicle fusion. In addition, they have shown that there is a greater than 10-fold increase in the density of amiloride-blockable Na\(^+\) channels within the apical membrane of the bladder in response to stretch. Based on these data, Lewis and de Moura (25, 26) postulated that during urinary bladder distension, there is a fusion of Na\(^+\) channel-containing cytoplasmic vesicles to apical membrane.

In summary, we have demonstrated that \(\alpha\)-, \(\beta\)-, and \(\gamma\)-ENaC subunits are expressed in the rat urinary bladder. Expression of ENaC in the urinary bladder suggests that the ENaC gene family represents the amiloride-sensitive Na\(^+\) conductance previously characterized in the mammalian urinary bladder by electrophysiological methods (13, 24, 26–28, 35).

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Address for reprint requests: T. R. Kleyman, Medical Research (151), Veterans Affairs Medical Center, University and Woodland Ave., Philadelphia, PA 19104.

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