Immunocytochemical and immunoelectron microscopic localization of α-, β-, and γ-ENaC in rat kidney

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Hager, Henrik, Tae-Hwan Kwon, Anna K. Vinnikova, Shyama Masilamani, Heddwen L. Brooks, Jørgen Frøkiaer, Mark A. Knepper, and Søren Nielsen. Immunocytochemical and immunoelectron microscopic localization of α-, β-, and γ-ENaC in rat kidney. Am J Physiol Renal Physiol 280: F1093–F1106, 2001.—Epithelial sodium channel (ENaC) subunit (α, β, and γ) mRNA and protein have been localized to the principal cells of the connecting tubule (CNT), cortical collecting duct (CCD), and outer medullary collecting duct (OMCD) in rat kidney. However, the subcellular localization of ENaC subunits in the principal cells of these cells is undefined. The cellular and subcellular localization of ENaC subunits in rat kidney was therefore examined. Immunocytochemistry demonstrated the presence of all three subunits in principal cells of the CNT, CCD, OMCD, and IMCD. In cortex and outer medulla, confocal microscopy demonstrated a difference in the subcellular localization of subunits. α-ENaC was localized mainly in a zone in the apical domains, whereas β- and γ-ENaC were found throughout the cytoplasm. Immunoelectron microscopy confirmed the presence of ENaC subunits in both the apical plasma membrane and intracellular vesicles. In contrast to the labeling pattern seen in cortex, α-ENaC labeling in IMCD cells was distributed throughout the cytoplasm. In the urothelium covering pelvis, ureters, and bladder, immunoperoxidase and confocal microscopy revealed differences the presence of all ENaC subunits. As seen in CCD, α-ENaC was present in a narrow zone near the apical plasma membrane, whereas β- and γ-ENaC were dispersed throughout the cytoplasm. In conclusion, all three subunits of ENaC are expressed throughout the collecting duct (CD), including the IMCD as well as in the urothelium. The intracellular vesicular pool in CD principal cells suggests ENaC trafficking as a potential mechanism for the regulation of Na⁺ reabsorption.

aldosterone; collecting duct; urothelium; epithelial sodium channel; intracellular trafficking; sodium transport

In several tight epithelia, electrogenic entry of Na⁺ from the lumen into the cells is mediated by the epithelial sodium channel (ENaC) located in the apical plasma membrane. This represents the rate-limiting step for Na⁺ absorption and is characterized by inhibition with submicromolar concentrations of the diuretic amiloride (11). On the basolateral side, the Na-K-ATPase actively transports sodium out of the cell into the extracellular interstitium and provides the driving force for Na⁺ absorption (11). ENaC, belonging to the degenerin/ENaC gene superfamily, is composed of three homologous subunits, α-, β-, and γ-ENaC.

ENaC is known to be present in several organs, including kidney, where ENaC subunits are known to be expressed in the connecting tubule (CNT), cortical collecting duct, (CCD), and outer medullary (OMCD) collecting duct (7, 21). In these segments ENaC participates in the fine regulation of Na⁺ reabsorption mediated by the hormones controlling sodium and water balance, e.g., the mineralocorticoid aldosterone and vasopressin (8, 12, 18, 21). This Na⁺ reabsorption via ENaC is electrogenic, hence creating a lumen negative gradient that promotes the passive chloride reabsorption via the paracellular pathway (6, 34) as well as the secretion of potassium into the lumen (29). The latter may also be enhanced by the increased activity of Na-K-ATPase mediated by the mineralocorticoid as well as by the aldosterone-sensitive K⁺ channels in the apical membranes (37).

Perhaps owing to the high turnover number of ENaC, physiological rates of sodium transport may occur in the collecting duct with relatively few copies of the ENaC complex compared with, for example, the collecting duct water channel aquaporin-2 (AQP2). The relatively low abundance of ENaC in the collecting duct cells has added to the challenge of discovering how its activity is regulated at the level of ENaC protein because standard-quality antibodies may have diffi-

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difficulty in demonstrating labeling above background levels. Recently, we have developed a set of antibodies to the three ENaC subunits that appear to offer an improvement in labeling efficiency in immunoblots and immunocytochemistry (21). In this paper, we exploit these antibodies in high-resolution light microscopic as well as electron microscopic localizations of ENaC in the kidney to provide new information regarding the cellular and subcellular localization of all three subunits along the collecting duct system. Specifically in this study, we have focused on the following four major objectives: 1) to define the localization of each of the three ENaC subunits along the axis at the collecting duct, including evaluating whether ENaC subunits are present in the inner medullary collecting duct (IMCD); 2) to define the subcellular localization by confocal microscopy to evaluate whether there is axial heterogeneity in the subcellular distribution of ENaC subunits; 3) to evaluate by confocal microscopy whether there are significant differences in the subcellular distribution of each subunit within the same cell; and 4) to define the subcellular localization of ENaC subunits by immunoelectron microscopy to establish the hypothesis that ENaC is present in both the apical plasma membrane and in the intracellular vesicles. Its presence in vesicles would be compatible with the view that ENaC regulation may involve regulated trafficking, as seen with vasopressin regulation of AQP2. These objectives were explored through the use of immunoblotting, immunocytochemistry, laser confocal microscopy, and immunoelectron microscopy.

METHODS

Antibody against ENaC subunits, AQP2, and H⁺-ATPase. Antibodies raised in rabbits against synthetic peptides were used. Affinity-purified polyclonal antibodies against α, β, and γ-ENaC (LL766AP, LL558AP, and LL550AP, respectively) have been described previously (21). An additional antisera against α-ENaC was also raised. For this, a peptide from the cytoplasmic NH₂-terminus of rat α-ENaC (4), NH₂-KGDKREEQGLGPEPSAPRQPC-COOH, corresponding to amino acids 48–67 with an additional cysteine at the COOH terminus, was synthesized, coupled to keyhole limpet hemocyanine, and used for immunization of rabbits by using a standard immunization protocol. Both immune serum and antibody-antigen reaction were visualized by using luminol-based enhanced chemiluminescence (LumiGLO, Kirkegaard and Perry Laboratories, Gaithersburg, MD) used at a concentration of 0.1 mg/ml. Sites of antibody-antigen reaction were visualized.

Immunelectron and immunocytochemistry. Kidneys from normal Munich-Wistar rats (n = 5) were fixed by retrograde perfusion via the aorta with 2% paraformaldehyde, in 0.1 M cacodylate buffer, pH 7.4. For semithin sections (0.8–1 μm), tissue blocks prepared from cortex, outer and inner stripes of outer medulla, and inner medulla were cryoprotected with 2.3 M sucrose containing 2% paraformaldehyde, mounted on holders, and rapidly frozen in liquid nitrogen (27). For immunoperoxidase and immunofluorescence microscopy, the paraffin-embedded tissues were cut into 2-μm sections on a rotary microtome (Micron). The staining was carried out by using indirect immunofluorescence or indirect immunoperoxidase. The sections were dewaxed and rehydrated. For immunoperoxidase labeling, endogenous peroxidase was blocked by 0.5% H₂O₂ in absolute methanol for 10 min at room temperature. To reveal antigens, sections were put in 1 mM Tris solution (pH 9.0) supplemented with 0.5 mM EGTA and heated by using a microwave oven for 10 min. Nonspecific binding of Ig was prevented by incubating the sections in 50 mM NH₄Cl for 30 min, followed by blocking in PBS supplemented with 1% BSA, 0.05% saponin, and 0.2% gelatin. Sections were incubated overnight at 4°C with primary antibodies diluted in PBS supplemented with 0.1% BSA and 0.3% Triton X-100. After rinsing with PBS supplemented with 0.1% BSA, 0.05% saponin, and 0.2% gelatin for 3 × 10 min, the sections for laser confocal microscopy were incubated in Alexa 488-conjugated goat anti-rabbit antibody (Molecular Probes) diluted in PBS supplemented with 0.1% BSA and 0.3% Triton X-100 for 60 min at room temperature. For double labeling, Alexa 546-conjugated goat anti-mouse antibody (Molecular Probes) was added as well. After rinsing with PBS for 3 × 10 min, the
sections were mounted in glycerol supplemented with anti-
fade reagent (N-propyl gallat). For immunoperoxidase, the
sections were washed (see above) followed by incubation
in horseradish peroxidase-conjugated goat anti-rabbit Ig
(DAKO P448) diluted in PBS supplemented with 0.1% BSA
and 0.3% Triton X-100. The microscopy was carried out by
using a Leica DMRE light microscope and a Zeiss LSM510
laser confocal microscope.

Immunoelectron microscopy. For immunoelectron micros-
copy, the frozen samples were freeze-substituted in a
Reichert AFS freeze substitution unit (16, 27, 38). In brief,
the samples were sequentially equilibrated over 3 days in
methanol containing 0.5% uranyl acetate at temperatures
gradually raised from −80 to −70°C, then rinsed in pure
methanol for 24 h while the temperature was increased from
−70 to −45°C, and infiltrated with Lowicryl HM20 and
methanol 1:1, 2:1 and, finally, pure Lowicryl HM20 before
ultraviolet polymerization for 2 days at −45°C and 2 days at
0°C. Immunolabeling was performed on ultrathin Lowicryl
HM20 sections. Sections were pretreated with a saturated
solution of NaOH in absolute ethanol (2–3 s), rinsed, and
preincubated for 10 min with 0.1% sodium borohydride and
50 mM glycine in 0.05 M Tris (pH 7.4) supplemented with
0.1% Triton X-100. Sections were rinsed and incubated over-
night at 4°C with antibodies diluted in 0.05 M Tris (pH 7.4)
supplemented with 0.1% Triton X-100 with 0.2% milk. After
rinsing, sections were incubated for 1 h at room temperature
with goat anti-rabbit IgG conjugated to 10-nm colloidal gold
particles (1:50, GAR.EM10, BioCell Research Laboratories,
Cardiff, UK). The sections were stained with uranyl acetate
and lead citrate before examination in Philips CM100 or
Philips 208 electron microscopes. Immunolabeling controls
were performed by using preabsorption of the immune serum
with the peptide used for immunization.

RESULTS

Immunoblotting of α-, β-, and γ-ENaC in rat kidney.
Immunoblotting of fractions prepared from rat kidney
homogenates by differential centrifugations (Fig. 1)
revealed that the affinity-purified antibodies directed
to each of the three ENaC subunits recognize a single
band of apparent molecular mass in the 83- to 90-kDa
range in renal membranes but not in cytosol (200,000-g
supernatant). Membrane fractionation revealed the
presence of all three ENaC subunits in the membrane
fractions that were enriched for either plasma mem-
branes or intracellular vesicles. The bands were not
present with purified IgG from preimmune serum from
the same rabbits.

To examine the presence of ENaC subunits in the
different zones of rat kidney, immunoblots of homoge-
nates from the rat whole kidney, cortex, outer medulla,
and three different zones of the inner medulla (base,
middle, and tip) were performed by using the same
affinity-purified rabbit anti-rat antibodies (Fig. 2, ar-
rowheads). In all the investigated zones of the rat
kidney including inner medulla, the antibodies labeled
distinct bands, corresponding to the three ENaC sub-
units (Fig. 2). This indicated that α-, β- and γ-subunits
of the ENaC are present in all kidney zones including
inner medulla. The relative abundance of each subunit
appears to decrease toward the inner part of the inner
medulla, however. The faint bands in the immunobLOTS
of β- and γ-ENaC (Fig. 2, tip of inner medulla) may

Fig. 1. Immunoblotting of α- (top), β- (middle), and γ-subunits (bot-
tom) of the epithelial sodium channel (ENaC) in rat kidney fractions
enriched for plasma membranes (17,000-g pellet), intracellular ves-
icles (200,000-g pellet), or cytoplasmic proteins (200,000-g superna-
tant). Twenty micrograms were loaded in each lane. Left: labeling
with affinity-purified antibodies. Right: labeling with protein A-pu-
rified IgG prepared from preimmune sera. Equal concentrations of
IgG were used for antibody and preimmune serum in each case.
tibodies were negative in all zones of the kidney (Fig. 4, Immunolabeling controls with peptide-preabsorbed an-
also exhibited significant labeling of shown). Importantly, the principal cells of the IMCD was found all the way to the papillary tip (Fig. 3, (Fig. 3, G–L), arrowheads) as well as in the OMCD (not shown), and inner medulla (Fig. 6, D–F). In contrast to the labeling pattern seen in cortex (with α-ENaC labeling restricted to the apical part of the principal cells), α-ENaC labeling in IMCD principal cells was distributed more evenly in the cytoplasm (cf. Fig. 6, A and D). In the outer medulla the labeling pattern of α-ENaC is more similar to the pattern seen in CCD, although slightly less polarized (not shown). Labeling of β- and γ-ENaC in the IMCD was similar to that seen in CCD (cf. Fig. 6, B–C and E–F).

Immunoelectron microscopy of the ENaC in collecting duct principal cells. Immunoelectron microscopy was performed by using immunogold labeling of sections prepared from kidney tissue embedded in Lowi-cryl HM20 by cryosubstitution. In sections from the kidney cortex, immunogold labeling of the β-subunit of the ENaC was observed of principal cells in the CNT (Fig. 7, PC (arrows)), whereas intercalated cells exhibited no immunogold labeling (Fig. 7, IC). In the principal cells, immunolabeling of the β-subunit was predominantly associated with intracellular vesicles (Figs. 7 and 8, arrows), some of which are clearly revealed in the section whereas others are more difficult to see because of weak fixation and/or tangential sectioning. Also, distinct labeling of the apical plasma membrane was seen (Fig. 8, arrowheads). This immunolabeling pattern was consistent with the confocal microscopic observations, demonstrating mainly intracellular labeling of the β-subunit (Figs. 5G and 6B). Consistently, immunogold labeling of the γ-subunit was also mainly associated with intracellular vesicles (Fig. 9, A–C), but some labeling was also seen associated with the apical plasma membrane domains (Fig. 9A, arrowhead) in kidneys of rats with no sodium restriction and no aldosterone treatment.

Fig. 2. Immunoblotting of α- (top), β- (middle), and γ-ENaC (bottom) in rat kidney. Immunoblots of membrane fractions from the rat kidney cortex (CTX), outer medulla (OM), and 3 different zones of the inner medulla (base (1), middle (2), and tip (3)) using the affinity-purified rabbit anti-rat antibodies against α-, β-, and γ-ENaC subunits, respectively. In all the investigated zones of the rat kidney including inner medulla, each antibody labeled a distinct band at ~83–90 kDa in membrane fractions.

represent modified β- and γ-ENaC, e.g., ubiquinated or otherwise posttranslationally modified.

Localization of α-, β-, and γ-ENaC in rat kidney by using immunohistochemistry. In both kidney cortex and outer medulla, immunoperoxidase microscopy using paraffin-embedded rat kidney tissues demonstrated that immunolabeling of the α-, β- and γ-subunits of ENaC was exclusively associated with principal cells in the CCD (Fig. 3, A–F and D–F, arrows) as well as in the OMCD (not shown). In contrast, intercalated cells exhibited no labeling of ENaC subunits in the CCD (Fig. 3, D–F, arrowheads) as well as in the OMCD (not shown). Importantly, the principal cells of the IMCD also exhibited significant labeling of α-, β-, and γ-subunits (Fig. 3, G–L), with decreasing labeling along the axis. A faint but distinct labeling of the ENaC subunits was found all the way to the papillary tip (Fig. 3, G–L). Immunolabeling controls with peptide-preabsorbed antibodies were negative in all zones of the kidney (Fig. 4, A–F).

Cellular and subcellular localization of α-, β-, and γ-ENaC subunits determined by single- and double-labeling confocal laser microscopy. To evaluate the cellular and subcellular localization of α-, β-, and γ-ENaC subunits, single- and double-labeling confocal microscopy was undertaken, and the labeling pattern was compared with that of AQP2 and H+-ATPase. AQP2 labeling was restricted to principal cells (Fig. 5, A and C), whereas H+-ATPase labeling was restricted to intercalated cells of the collecting duct (Fig. 5, B–C). Double labeling using antibodies against α-, β-, and γ-ENaC subunits and H+-ATPase revealed that α- (Fig. 5, D and F), β- (Fig. 5, G and I), and γ-ENaC (Fig. 5, J and L) were exclusively localized in the principal cells (arrows), consistent with previous results. Importantly, confocal microscopy revealed that in CNT (not shown) and CCD, α-ENaC labeling was exclusively restricted to a narrow zone in the apical part of the principal cells including the plasma membrane domains (Fig. 5, D and F, arrows). Compared with the labeling of AQP2 (in neighboring sections; see Fig. 5, A and C), α-ENaC labeling was considerably more re-

restricted to the very apical part of the principal cells. In contrast, β- and γ-subunits were observed to be distributed throughout the cytoplasm with no distinct increased labeling in the apical domains of the principal cells (Fig. 5, G and I and J and L (arrows), respectively).

To evaluate whether there was an axial heterogene-

ity in the subcellular localization of ENaC subunits along the axis of the collecting duct, confocal laser microscopy was performed in cortex (Fig. 6, A–C), outer medulla (not shown), and inner medulla (Fig. 6, D–F). In contrast to the labeling pattern seen in cortex (with α-ENaC labeling restricted to the apical part of the principal cells), α-ENaC labeling in IMCD principal cells was distributed more evenly in the cytoplasm (Fig. 6, A and D). In the outer medulla the labeling pattern of α-ENaC labeling was similar to that seen in the inner medulla (cf. Fig. 6, B–C and E–F).

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Fig. 3. Immunoperoxidase microscopy of α-, β-, and γ-ENaC subunits using paraffin-embedded rat kidney tissues. A–C: Immunolabeling of the α-, β-, and γ-ENaC subunits are exclusively associated with principal cells in the cortical collecting ducts (CCD), respectively. G, glomerulus. D–F: High magnification reveals that ENaC subunits are exclusively associated with principal cells in the CCD (arrows), whereas intercalated cells exhibited no labeling (arrowheads). G–I: In the inner medulla, principal cells also exhibit a significant labeling of α-, β-, and γ-ENaC, respectively. J–L: High magnification reveals a distinct labeling of the ENaC subunits in the terminal collecting duct [inner medullary collecting duct (IMCD)] principal cells. Magnification: ×220 (A–C, G–I); ×550 (D–F, J–L).
labeling of α-ENaC revealed weak labeling of intracellular vesicles and apical plasma membrane close to background levels (not shown). Thus immunoelectron microscopy demonstrates that ENaC subunits are present in both intracellular vesicles and the apical plasma membrane.

Localization of α-, β- and γ-subunits of ENaC in urothelium. A marked ENaC subunit labeling of the transitional epithelium (urothelium) covering the renal pelvic wall was also found (Fig. 10). Consistent with the difference in the subcellular localization in the CCD principal cells, both immunoperoxidase (Fig. 10, A, C, and E) and confocal microscopy (Fig. 10, B, D, and F) revealed that α-ENaC was located in and/or near the apical plasma membranes (Fig. 10, A and B), whereas β (Fig. 10, C and D)- and γ-ENaC (Fig. 10, E and F) were located throughout the cytoplasm in a diffuse pattern. The umbrella cells were seen to be devoid of α-ENaC, whereas they expressed β- and γ-ENaC. This distribution pattern was also seen in urothelium of the urinary bladder (not shown). Immunolabeling controls were negative (Fig. 4, G–I).

DISCUSSION

We have demonstrated that α-, β-, and γ-ENaC are present not only in the principal cells of the CNT, CCD, and OMCD but are also abundantly expressed in principal cells of the IMCD, consistent with previous physiological data from isolated perfused IMCD (17). Thus
AQP2 (C)

H⁺-pump (C)

α-ENaC (E)

H⁺-pump (E)

β-ENaC (I)

H⁺-pump (I)

γ-ENaC (L)

H⁺-pump (L)

Fig. 5. Colocalization of aquaporin-2 (AQP2; C), α-ENaC (F), β-ENaC (I), and γ-ENaC (L) with H⁺-ATPase in the CCD. A–C: AQP2 is present at the apical part of the principal cells (A and C, arrows), whereas H⁺-ATPase is present at the apical part of the type-A intercalated cells (B and C, arrowheads). Thus AQP2 is exclusively expressed in the principal cells (C, arrows), whereas H⁺-ATPase is located at the intercalated cells (C, arrowheads) in the same duct. D–L: α (D and F, arrows), β (G and I, arrows), and γ-ENaC (J and L, arrows) are exclusively localized at the principal cells. In contrast, H⁺-ATPase is located only at the intercalated cells (E, H, and K, arrowheads). Confocal microscopy also reveals that the α-ENaC is mainly localized at the apical portion of the principal cells (D and F, arrows) in contrast to β- and γ-ENaC (G and I and J and L, respectively). Magnification: ×800 (A–L).
all three subunits of ENaC are present along the entire collecting duct. Moreover, we have demonstrated that the subcellular localization of the three ENaC subunits is distinctly different and also differs along the axis of the collecting duct. Laser confocal microscopy demonstrated that α-ENaC is predominantly present in the extreme apical domains of the principal cells in CCD and OMCD. In contrast, labeling of β- and γ-ENaC is mainly associated with intracellular vesicles dispersed in the entire cytoplasm with no increase in labeling in the apical plasma membrane domains. The differences in the subcellular localization among α-, β-, and γ-ENaC subunits suggest that there are some differences in the regulation of ENaC subunits. In contrast to the difference in the subcellular localization of α-ENaC vs. β- and γ-ENaC in CCD and OMCD, there was virtually no difference in the subcellular localization of all three subunits in IMCD, where most labeling was observed in cytoplasmic areas. Thus there is an axial heterogeneity in the subcellular localization of α-ENaC. Immunelectron microscopy demonstrated that ENaC subunits are present in both the apical plasma membrane and intracellular vesicles, consistent with the possibility that ENaC regulation may involve trafficking from vesicles to the apical plasma membrane, analogous to vasopressin regulation of AQP2 trafficking. Finally, we demonstrated that all three ENaC subunits were localized in the urothelium covering the proximal portions of the urinary tract, thereby extending previous studies showing the presence of ENaC in bladder urothelial cells (33). Again, there was a distinct difference in the subcellular localization because α-ENaC (in contrast to β- and γ-ENaC) was localized in a highly polarized fashion, in a narrow region in the apical section of the cells. Moreover, in the superficial cell layer (the umbrella cells) only β- and γ-ENaC were present, demonstrating that α-ENaC may not be uniformly present in all cells expressing β- and γ-ENaC.

ENaC localization in the renal collecting duct. The present observation that α-, β-, and γ-ENaC are expressed in principal cells of the IMCD is consistent with the possibility that ENaC regulation may involve trafficking from vesicles to the apical plasma membrane, analogous to vasopressin regulation of AQP2 trafficking. Finally, we demonstrated that all three ENaC subunits were localized in the urothelium covering the proximal portions of the urinary tract, thereby extending previous studies showing the presence of ENaC in bladder urothelial cells (33). Again, there was a distinct difference in the subcellular localization because α-ENaC (in contrast to β- and γ-ENaC) was localized in a highly polarized fashion, in a narrow region in the apical section of the cells. Moreover, in the superficial cell layer (the umbrella cells) only β- and γ-ENaC were present, demonstrating that α-ENaC may not be uniformly present in all cells expressing β- and γ-ENaC.

Fig. 6. Confocal microscopy using immunofluorescence labeling of ENaC subunits in sections from kidney cortex (A–C) or from inner medulla (D–F). A–C: a significant labeling of α-, β-, and γ-ENaC subunits is exclusively associated with principal cells of the collecting ducts (arrows), whereas intercalated cells were not labeled. α-ENaC is mainly localized at the apical portion of the principal cells (A, arrows). Arrowheads, basal portions of the principal cells. In contrast, labeling of β- and γ-subunit is observed as dispersed throughout the cytoplasm with no distinct labeling at the apical part of the cells (B and C, arrows, respectively). D–F: in the inner medulla, principal cells also exhibit a significant labeling of α-, β-, and γ-subunit, respectively. Magnification: ×1,000 (A–C); ×630 (D–F).
with physiological data demonstrating amiloride-inhibited sodium conductance in the apical membrane of isolated and perfused rat IMCD (17). One of the early studies on ENaC expression in kidney (7) reported that ENaC was not present in the IMCD and suggested that another channel would be involved in sodium reabsorption at this site, whereas earlier immunocytochemical works demonstrated ENaC in rat IMCD (3, 36). Our data showing the presence of all three subunits in IMCD strongly support the view that ENaC subunits are involved in IMCD sodium reabsorption. The discrepancy between the two studies is likely to be due to differences in the level of expression along the collecting duct axis. As demonstrated in this study, there is indeed an axial heterogeneity in ENaC expression levels because ENaC immunolabeling is somewhat stronger in the CCD and OMCD compared with IMCD. Thus the lack of identification of ENaC in IMCD in the previous study may simply be a matter of detection level. In support of this view, we optimized the labeling procedures in this study, which resulted in a 3- to 10-fold increase in labeling efficiency (with conventional methods, 3–10 times higher antibody concentration was necessary to achieve labeling). Thus the presence of ENaC also in the IMCD supports the view that ENaC is also involved in amiloride-sensitive sodium reabsorption at this site.

Different subcellular localization of α- vs. β- and γ-ENaC subunits in collecting duct principal cells. ENaC consists of at least three structurally related subunits (α-, β-, and γ-ENaC), and all three subunits participate in channel formation as the absence of any

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Fig. 7. Immunoelectron microscopy of the β-ENaC subunit in the CCD in ultrathin Lowicryl HM20 sections. Immunogold labeling of the β-ENaC subunit in principal cells (PC, arrows) is observed, whereas intercalated cells (IC) exhibit no immunogold labeling. In the PC, immunolabeling of the β-subunit is predominantly associated with intracellular vesicles (arrows). Magnification: ×63,000.
one results in a significant reduction of sodium current expression in *Xenopus laevis* oocytes. We demonstrated by immunoblotting, immunocytochemistry, confocal microscopy, and immunoelectron microscopy that ENaC subunits are present in both intracellular vesicles and the apical plasma membrane. Moreover, we have demonstrated that immunolabeling of α-ENaC is mainly present at the apical domains of the principal cells, whereas the labeling of β- and γ-ENaC is associated with intracellular vesicles dispersed in the entire cytoplasm, with sparse labeling of the apical plasma membranes. This may suggest that there are some differences in the regulation of ENaC subunits. Previous studies have also shown discrepancies with regard to the three subunits. Increased plasma aldosterone levels, induced by either sodium restriction or aldosterone infusion, were associated with a marked increase in the abundance of α-ENaC protein in kidneys, but the abundance of the β- and γ-ENaC subunit proteins did not increase (21). Consistent with this, α-ENaC appears to be the only subunit of the three that has been reported to be transcriptionally regulated by aldosterone (1, 9, 28, 35). Moreover, 7-day water restriction of Sprague-Dawley rats resulted in significantly increased abundances of β- and γ-ENaC but no change in α-ENaC, whereas chronic vasopressin infusion increased the abundance of the all three ENaC subunit proteins (8). The implication of changes in abundance of one or two subunits and not the other(s) in sodium transport capacity is not known. Because the synthesis of the α-ENaC subunit has been suggested to be a rate-limiting factor of the multimeric ENaC complex
sodium transport could be expected to be proportional to the abundance of the α-ENaC protein levels. Further studies are needed to assess the impact of differential regulation and differential subcellular localization of the ENaC subunits on sodium transport. As discussed above, α-ENaC labeling in the IMCD reveals enhanced vesicular labeling (in addition to labeling of plasma membrane domains) compared with CCD and OMCD [interestingly, this is exactly similar to the labeling pattern of AQP2 (see Fig. 5, for example), which also shows this segmental difference]. The higher molecular weight band in the immunoblot is likely to reflect important issues of ENaC biology. The top band in the immunoblot of β-ENaC (Fig. 2) may represent modified β-ENaC, i.e., ubiquinated or otherwise modified. Moreover, immunoblotting of γ-ENaC reveals a second, higher molecular weight band in the middle portion and tip of the inner medulla (Fig. 2). The reason is unknown but is likely to reflect chemical modification of the subunit.

Mechanisms of regulation of sodium reabsorption in collecting duct by ENaC. Renal regulation of sodium reabsorption or excretion is essential to the regulation of extracellular fluid volume as well as to the control of blood pressure. Sodium is reabsorbed in the different renal tubular segments through several renal sodium transporters: in proximal tubule, type 3 Na\(^+\)/H\(^+\) exchanger (NHE3) and type II Na\(^+\)-Pi cotransporter (NaPi-2) are both expressed apically (2, 23), whereas Na-K-ATPase is heavily expressed in the basolateral membrane of renal tubule cells, and are responsible for sodium reabsorption (32). The loop of Henle generates a high osmolality in renal medulla by driving the countercurrent multiplier, which is dependent on NaCl absorption by the thick ascending limb (TAL). The apically expressed Na-K-2Cl cotransporter (rat type 1 bumetanide-sensitive cotransporter) and NHE3, in conjunction with basolaterally expressed Na-K-ATPase are mainly responsible for sodium reabsorption by the TAL (15). In the distal convoluted tubule, the thiazide-sensitive Na-Cl cotransporter is involved in apical sodium reabsorption (14). As described above, regulation of sodium reabsorption also occurs in the CNT and collecting duct, and ENaC plays a critical role in this. The importance of ENaC in volume regulation has been demonstrated in recent studies that have identified mutations in ENaC as the basis of the pathogenesis of Liddle’s syndrome, a disorder characterized by volume expansion and hypertension (13, 31) as well as type I pseudohypoaldosteronism, a disorder characterized by volume depletion and hypotension (5). ENaC is regulated by the adrenal mineralocorticoid hormone vasopressin and insulin, which markedly increase the apical permeability of the collecting duct to sodium.

In principle, there may be at least two mechanisms for regulation of ENaC activity in the apical plasma membrane. One may involve regulation of ENaC expression, and in addition regulated trafficking of ENaC subunits may be involved, analogous to vasopressin regulation of AQP2 (i.e., short-term regulation of trafficking and long-term regulation of expression). Masiclamani et al. (21) recently demonstrated by immuno-

Fig. 10. Immunoperoxidase (A, C, and E) and confocal microscopy (B, D, and F) of ENaC subunits in the surface epithelium (urothelium) covering the pelvis. α-ENaC is located in and/or near the apical plasma membranes (A and B), whereas β (C and D)- and γ-ENaC (E and F) were located throughout the cytoplasm in a diffuse pattern. Magnification ×630.
fluorescence microscopy that sodium restriction in rats was associated with a redistribution of diffuse intracellular labeling of ENaC subunits to the apical regions of the principal cells as well as a marked increase in α-ENaC labeling (21). Similar observations have been recently reported by Loffing et al. (19). They suggested that ENaC protein may be regulated by both intracellular trafficking to the apical plasma membrane domains (short-term regulation) and by increasing protein abundance (long-term regulation) in response to aldosterone or other stimuli for enhancing sodium re-absorption in the distal nephron. Our study showing ENaC subunits in both the apical plasma membrane and intracellular vesicles is consistent with the possibility of regulated trafficking from vesicles to the apical plasma membrane. Future studies will be aimed at defining whether ENaC subunits undergo significant regulated trafficking in response to aldosterone, vasopressin, or other hormones.

Localization of ENaC subunits in pelvic urothelial cells. Our data demonstrate that all three subunits of ENaC are expressed in urothelial cells covering the renal pelvis. This extends previous data showing the presence of ENaC in bladder urothelium (33). Two observations in particular are curious. First, there is a pronounced difference in the subcellular distribution of α-ENaC vs. β- and γ-ENaC. This is similar to what is seen in the CCD and OMCD, which is that α-ENaC has a pronounced polarized distribution with labeling of the plasma membrane domains and very little labeling within the cell. In contrast, both β- and γ-ENaC are distributed in a dispersed vesicular pattern in the cytoplasm. Second, there is a complete absence of β-ENaC immunolabeling in the outer cell layer (the umbrella cells) whereas significant labeling of β- and γ-ENaC was observed. This raises the possibility that ENaC subunits may be separately expressed and the potentiality that additional isoforms exist to replace α-ENaC. The role of ENaC at this site and potential regulation of ENaC in the urothelial cells will require additional analysis.

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