Surface Expression of Epithelial Na Channel Protein in Rat Kidney

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Expression of epithelial Na channel (ENaC) protein in the apical membrane of rat kidney tubules was assessed by biotinylation of the extracellular surfaces of renal cells and by membrane fractionation. Rat kidneys were perfused in situ with solutions containing NHS-biotin, a cell-impermeant biotin derivative that attaches covalently to free amino groups on lysines. Membranes were solubilized and labeled proteins were isolated using neutravidin beads, and surface β and γENaC subunits were assayed by immunoblot. Surface αENaC was assessed by membrane fractionation. Most of the γENaC at the surface was smaller in molecular mass than the full-length subunit, consistent with cleavage of this subunit in the extracellular moiety close to the first transmembrane domains. Insensitivity of the channels to trypsin, measured in principal cells of the cortical collecting duct by whole-cell patch-clamp recording, corroborated this finding. ENaC subunits could be detected at the surface under all physiological conditions. However increasing the levels of aldosterone in the animals by feeding a low-Na diet or infusing them directly with hormone via osmotic minipumps for 1 wk before surface labeling increased the expression of the subunits at the surface by two- to fivefold. Salt repletion of Na-deprived animals for 5 h decreased surface expression. Changes in the surface density of ENaC subunits contribute significantly to the regulation of Na transport in renal cells by mineralocorticoid hormone, but do not fully account for increased channel activity.

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INTRODUCTION

The epithelial Na channel (ENaC) mediates Na reabsorption in distal portions of the nephron including the connecting tubule and collecting duct (Garty and Palmer, 1997; Kellenberger and Schild, 2002). These channels, and by extension distal nephron Na transport, are strongly regulated by the adrenal mineralocorticoid hormone aldosterone, and this regulation is crucial for maintenance of Na balance (Verrey et al., 2000). Genetic deletion or loss-of-function mutations in the channels leads to Na wasting and hypotension, while conversely, hyperactivity of the channels causes Na retention and hypertension (Lifton et al., 2001; Rossier et al., 2002).

Despite the importance of this regulatory system, the underlying cellular mechanisms are not completely understood. Although aldosterone-dependent Na transport depends on changes in transcription and translation of specific genes and proteins, increased synthesis of channel subunits themselves account for at most a minor component of the overall response of the mammalian kidney to the hormone (Asher et al., 1996; Masilamani et al., 1999; Ergonul et al., 2006). Another possibility involves alterations in channel protein trafficking leading to increased surface expression of the subunits. Consistent with this idea, immunocytochemical studies showed redistribution of β and γENaC protein from a diffuse intracellular to a more apical pattern in response to aldosterone (Masilamani et al., 1999; Loffing et al., 2000, 2001; Hager et al., 2001). In addition, one aldosterone-induced protein, SGK1, is thought to increase apical expression of ENaC by inhibition of ubiquitinylation and retrieval of protein from the surface (Debonneville et al., 2001; Snyder et al., 2002).

Canessa and colleagues used biotinylation of membrane surface proteins to show that aldosterone increased the amount of ENaC protein in the apical membrane of the amphibian renal cell line A6 (Alvarez de la Rosa et al., 2002). This was apparently due to increased rates of channel delivery to the surface and correlated with parallel increases in subunit synthesis (May et al., 1997; Alvarez de la Rosa et al., 2002) Similar measurements have not been made in mammalian renal cells, where at least in vivo the extent of regulation of the channels can be much greater while the role of channel biosynthesis is less important. We sought to fill this gap by adapting the biotinylation approach to the intact rat kidney. Here we show that aldosterone increases surface expression of ENaC subunits but that this may not account for the entire response to the hormone.

MATERIALS AND METHODS

Animals

All animal handling procedures were approved by the Institutional Animal Care and Use Committee of Weill-Cornell Medical College. Sprague-Dawley rats of either gender (Charles River Laboratories) were raised free of viral infections. Rats used for biotinylation experiments weighed 250–300 g. Those used for

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The online version of this article contains supplemental material.

Abbreviations used in this paper: CCD, cortical collecting duct; ENaC, epithelial Na channel; NCC, Na-Cl cotransporter.

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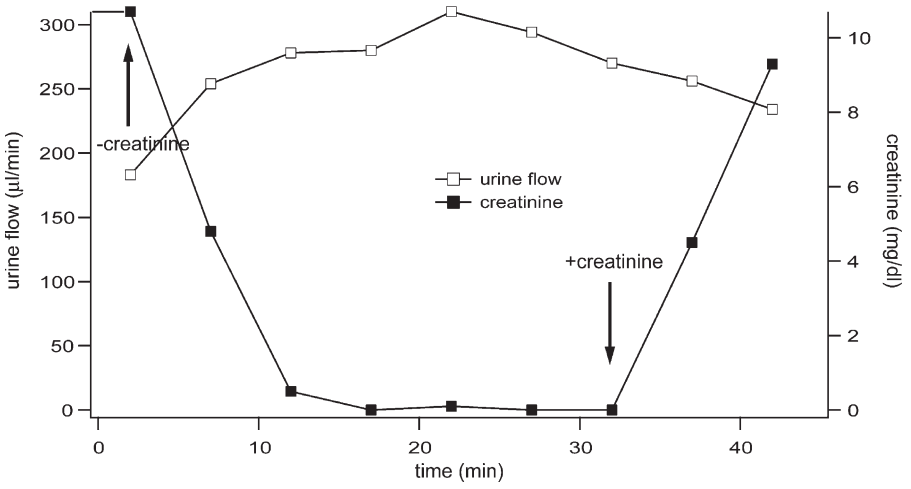

Figure 1. Urine flow and creatinine concentration in a kidney perfused at 5°C. Urine was collected from the left ureter. The perfusate initially contained 10 mg/dl creatinine, and the urine had a similar concentration. The perfusate was switched to a creatinine-free solution at $t = 2$ min and back to creatinine-containing solution at $t = 32$ min.

electrophysiological experiments were smaller (150–200 g). Animals were fed sodium-deficient rat diet (MP Biomedicals) or a modified diet that matches the low-Na diet but that contains 1% NaCl for 6–8 d. Some animals were fed the 1% NaCl diet and implanted subcutaneously with osmotic minipumps (model 2002, Alza Corp.) for 6–7 d to increase levels of circulating aldosterone. Aldosterone was dissolved in polyethyleneglycol 300 at 2 mg/ml to give a calculated infusion rate of 1 µg/h.

Biotinylation Procedure

For biotinylation of kidney cells rats were anesthetized with inactin (100 mg/Kg, i.p.) (Sigma-Aldrich) and the abdominal cavity was opened. The superior mesenteric artery and celiac trunk were ligated. The aorta was cannulated below the renal arteries and later ligated above the celiac trunk. The left renal vein was punctured to allow fluid outflow. The kidneys were perfused by gravity at a rate of ~10 ml/min with ice-cold solution. At this point the animal was killed by opening the chest and sectioning the cardiac apex. The rat was placed on an ice block and the kidneys were superfused with a steady stream of ice-cold PBS. Temperatures measured in the abdominal cavity were 7–9°C . In some experiments urine was collected from the left ureter via a catheter. In other cases flow rates through the urethra were observed and were ~0.2–0.5 ml/min.

The kidneys were perfused for 10–15 min with PBS containing heparin to clear the renal circulation of blood, then for 20–25 min with biotinylation solution, and finally for 30 min with biotin-quenching solution to stop its reaction with the tissue. The biotinylation solution was PBS with 4.5 mM KCl, pH 8.0, and 0.5 mg/ml sulfosuccinimidyl-2-[biotinamido]ethyl-1,3-dithiopropionate (sulfo-NHS-biotin, Pierce Chemical Co.). The quenching solution was PBS in which 25 mM TRIS-HCL, pH 8.0, replaced 25 mM NaCl.

The left kidney was removed on ice for further processing. In some cases the right kidney was also processed. The whole kidney was minced with a razor blade and homogenized with a tight-fitting Dounce in ice-cold lysis buffer containing 250 mM sucrose, 10 mM triethanolamine, pH 7.4, and protease inhibitors leupeptin (1 µg/ml) and PMSF (0.1 µg/ml). The homogenate was centrifuged for 10 min at 1,000 *g* to separate intact cells and nuclei. The supernatant was centrifuged at 18,000 *g* for 6 h to sediment a total membrane fraction. This pellet was suspended in 2.5 ml lysis buffer, aliquoted, and frozen at −80°C. Aliquots of this fraction containing 3 mg protein were solubilized in 0.5 ml buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, and 3% Triton-X100. This concentration of detergent solubilized all the detectable ENaC protein in the samples. This was added to 0.8 ml of a 50% suspension of neutravidin beads (Pierce Chemical Co.) and kept overnight at 4°C with gentle rocking. The beads were washed three times with wash buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, and 1% Triton X-100), twice with high-salt wash buffer (500 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, and 0.1% triton X-100), and twice with no-salt wash buffer (10 mM Tris-HCl, pH 7.4). The supernatant was decanted and protein bound to the beads was eluted in 60 µl 4× Laemmli sample buffer plus 25 µl of 0.5 M DTT for 15 min at 90°C. 10–25 µl of the eluate was loaded onto polyacrylamide gels for separation by SDS-PAGE.

For biotinylation of *Xenopus* oocytes, 20–30 oocytes injected with cRNA for α, β, and γENaC were incubated in 10 ml biotinylation solution as described above, diluted with one part H_2O to two parts solution, with or without biotin for 30 min at 4°C. The oocytes were washed with biotinylation quenching solution also diluted with one part H_20 to two parts solution for 30 min. The oocytes were then suspended in 10 µl/ooctye of lysis buffer (as above for kidneys) with 1% Triton-X100, broken by trituration with a Pasteur pipette and homogenized in a small Dounce. The homogenate was centrifuged at 10,000 *g* for 10 min and the biotinylated proteins in the supernatnant were isolated with neutravidin beads as described above.

Antibodies

Polyclonal antibodies against the α, β, and γ subunits of the rat ENaC were based on short peptide sequences in the amino terminus of αENaC and the carboxy termini of βENaC and γENaC as described previously (Masilamani et al., 1999; Ergonul et al., 2006). Antisera were purified using peptide-linked agarose bead affinity columns (Sulfolink Kit, Pierce Biotechnology). The basic characterization of these antisera was published previously (Ergonul et al., 2006). Other antibodies were obtained commercially: anti-Golgin from Calbiochem, anti-calnexin from Stressgen Bioreagents (AssayDesigns), and anti-NCC, NKCC2, and NHE3 from Chemicon International (Millipore Corp.).

Protein in membrane fractions was measured (BCA Kit, Pierce Biotechnology). Equal amounts of protein (40–50 µg/sample) were solubilized at 70°C for 10 min in Laemmli sample buffer and were resolved on 4–12% bis-Tris gels (Invitrogen) by SDS-PAGE. For immunoblotting, the proteins were transferred electrophoretically from unstained gels to PVDF membranes. After blocking with BSA, membranes were incubated overnight at 4°C with primary antibodies against α, β, or γENaC subunits at 1:500 or 1:1,000 dilutions. Anti-rabbit IgG conjugated with alkaline phosphatase was used as a secondary antibody. The sites of antibody–antigen reaction were visualized with a chemiluminescence substrate (Western Breeze, Invitrogen) before exposure to x-ray

film (Biomax ML, Kodak). Band densities were quantitated using a Quantity One densitometer and acquisition system (Bio-Rad Laboratories, Inc.).

Electrophysiology

For whole-cell clamp measurements, tubules were superfused with solutions prewarmed to 37°C containing (in mM) 135 Na methanesulfonate, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 2 glucose, 5 mM $BaCl_2$, and 10 HEPES adjusted to pH 7.4 with NaOH. The patch-clamp pipettes were filled with solutions containing (in mM) 7 KCl, 123 aspartic acid, 20 CsOH, 20 TEAOH, 5 EGTA, 10 HEPES, 3 MgATP, and 0.3 NaGDPβS with the pH adjusted to 7.4 with KOH. The total concentration of K^+ was ~120 mM. Pipettes were pulled from hematocrit tubing, coated with Sylgard, and fire polished with a microforge. Pipette resistances ranged from 2 to 5 MΩ. Amiloride-sensitive currents were measured as the difference in current with and without 10 µM amiloride in the bath solution. Trypsin (bovine pancreas) was obtained from Sigma-Aldrich. *Xenopus* oocytes expressing ENaC subunits were studied using two-electrode voltage clamp as described previously (Anantharam et al., 2006).

Differential Centrifugation

Plasma membrane–enriched fractions from kidney homogenates were prepared as described previously (Marples et al., 1995). Postnuclear (2,000 *g*) supernatants were centrifuged for 30 min at 17,000 *g*. The supernatant was centrifuged again at 17,000 *g* for 12 h. The postnuclear supernatants, 30-min pellets, and 12-h pellets were assayed for the plasma membrane marker enzyme K-dependent phosphatase (Murer et al., 1976) and for the endoplasmic reticulum marker enzyme NADPH-dependent cytochrome C reductase (Mircheff et al., 1979).

Online Supplemental Material

The online supplemental material (Figs. S1 and S2, available at http://www.jgp.org/cgi/content/full/jgp.200809989/DC1) contains immunoblots showing β and γENaC expression in cell fractions from the kidney obtained by differential centrifugation as described above.

RESULTS

We have adapted the technique of labeling surface proteins with biotin (Gottardi et al., 1995) to the intact organ. Our strategy was to label proteins in the apical membranes of the kidney epithelial cells using glomerular filtration to deliver biotin derivatives into the tubular lumen. For our purposes it was crucial to maintain cells at low temperature during exposure to biotin in order to prevent membrane protein trafficking. Preliminary experiments were therefore designed to see if the kidneys would maintain glomerular filtration while maintained at temperatures of <10°C. As shown in Fig. 1, urine flow through one kidney could be maintained above 200 µl/min. This is comparable to the normal GFR of rat kidney (Frindt et al., 2001) and is consistent with robust filtration and lack of significant fluid absorption under these conditions. It was also important to be able to alter the composition of the tubule fluid fairly quickly. We assessed this in the same preliminary experiments by adding and removing creatinine from the perfusate. This compound is freely filtered at the glomerulus and easily measured in the urine. As can be seen in Fig. 1, nearly complete wash-in or washout of creatinine to or from the collected urine required ~10 min of perfusion.

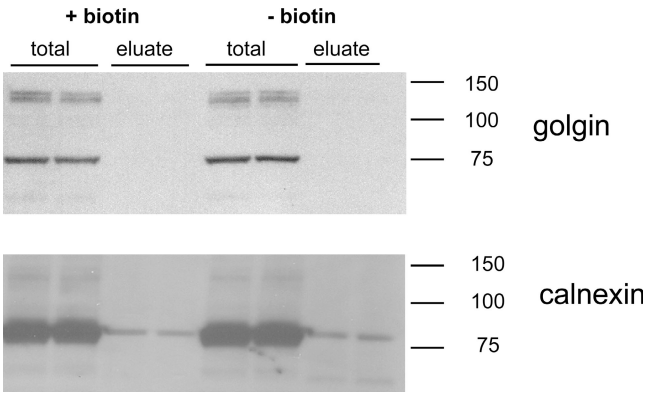

Figure 2. Absence of biotin labeling of intracellular membrane proteins. Kidneys were perfused with or without biotin. Lanes were loaded with 15 µg of total membrane protein or 20 µl of eluate from the neutravidin beads. Duplicate adjacent lanes represent separate material from each of two animals for each condition. The upper blot was stained with anti-golgin; the full length protein corresponds to the doublet of high apparent molecular mass. No staining of the eluates could be detected either with or without biotin. The lower blot was stained with anti-calnexin, which gave a very strong signal with the total membrane pool. Similar staining was observed with the eluates from kidneys perfused with or without biotin, indicating the absence of biotinylation of this protein. In these and subsequent blots, numbers to the right correspond to the positions of molecular mass standards in kD.

To test whether the biotin compound actually reached the distal part of the nephron where the Na channels are located, we also measured the biotin content of the urine during perfusion with biotin label. With a starting concentration of 500 µg/ml in the perfusate, we found an average of 340 µg/ml in the collected urine. Although there is some retention of label by the blood vessels and tubules, a significant fraction of the starting concentration reaches the distal nephron and beyond. Thus we concluded that it was feasible to label distal nephron membrane proteins with this approach.

We used sulpho-NHS-SS-biotin to label these proteins. The compound has a molecular mass of 600 D, permitting free filtration at the glomerulus. The probe is assumed to be membrane impermeant due to the presence of the negatively charged sulphonate moiety. To confirm this we tested for biotinylation of intracellular membrane proteins, as shown in Fig. 2. The top panel illustrates staining of total kidney membranes as well as eluates from the neutravidin beads, which represents biotinylated protein, with an antibody directed against golgin (GM130), a membrane protein expressed in the cis-Golgi apparatus (Marra et al., 2001). A clear signal was observed in total membranes but not in the eluates. As a second negative control we assayed for calnexin, a

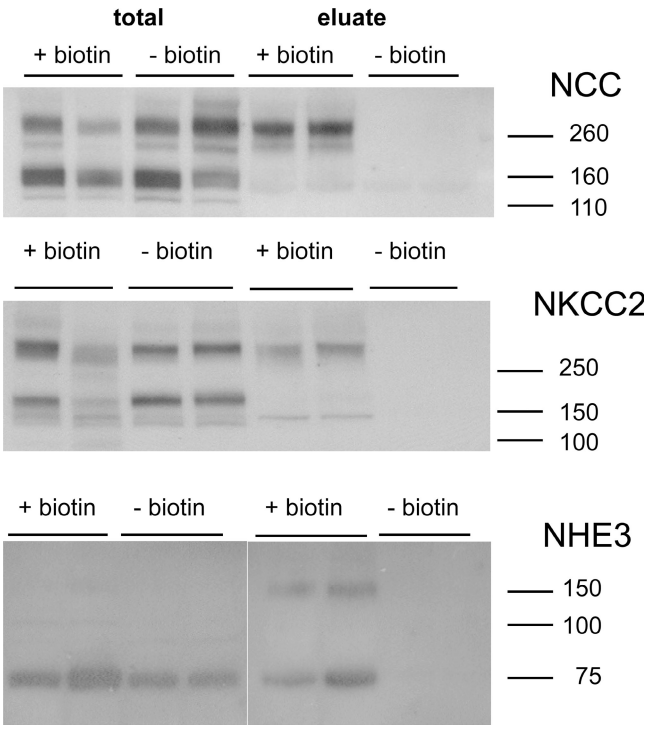

Figure 3. Biotinylation of plasma membrane proteins. Kidneys were perfused with or without biotin. Lanes were loaded with 45 μg of total membrane protein or 25 μl of eluate from the neutravidin beads. Top: Na/Cl cotransporter (NCC). In total membranes, the antibody reacted with proteins of apparent molecular masses of ∼130 and ∼260 kD, presumably reflecting monomeric and dimeric forms of the transporter. The material in the biotinylated eluate was mostly at the higher molecular mass. No staining was observed in the nonbiotinylated eluate. Middle: Na/K/2Cl cotransporter (NKCC2). Three bands were observed, with the major ones at ∼140 and ∼280 kD, again presumably reflecting monomeric and dimeric forms of the transporter. The sharper band at ∼110 kD may represent the nonglycosylated form of the protein. The eluate of the biotinylated kidneys contained primarily the 280-kD form. No staining could be detected in the eluate from the nonbiotinylated kidneys. Bottom: Na/H exchanger (NHE3). The total membrane fraction contained a predominant band at ∼80 kD. The eluate from the biotinylated kidneys also contained this presumably full-length form of the protein, and also had a species at ∼160 kD that may represent a dimer. No staining could be detected in the eluate from the nonbiotinylated kidneys. (Panel has been rearranged so that the lanes correspond to those of the top two panels.)

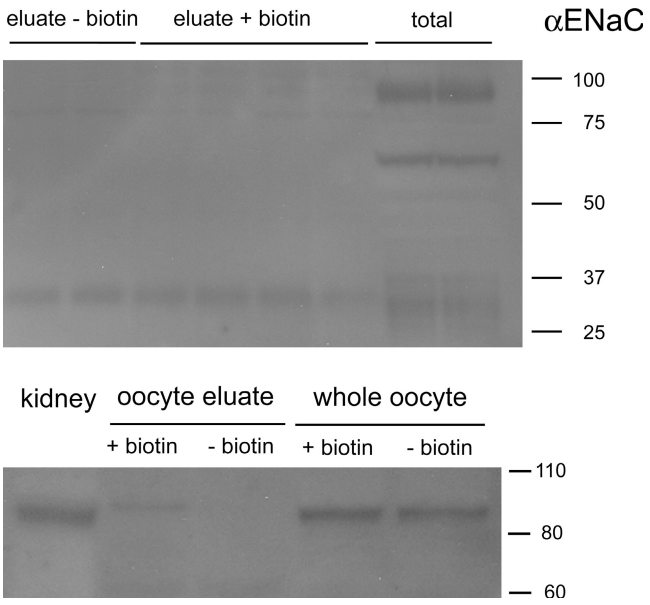

Figure 4. Biotinylation of αENaC in kidneys and oocytes. Top: kidneys from rats on a low-Na diet were perfused with or without biotin. Lanes were loaded with 40 μg of total membrane protein or 25 μl of eluate from the neutravidin beads. Total membranes from whole kidney had three stained bands at ∼90, ∼65, and ∼30 kD. Only the 30-kD band was observed in eluates from neutravidin beads, but staining was similar in biotinylated and nonbiotinylated kidneys, indicating nonspecific binding to neutravidin beads. Bottom: The major peptide in ENaC-expressing oocytes is the full-length 90-kD form. Faint staining of material at this molecular mass was observed in the eluate of biotinylated but not of nonbiotinylated oocytes, showing that the protein can react with the biotin reagent. Similar results were obtained with three independent batches of oocytes. The 30-kD peptide could not be resolved due to nonspecific staining of an unidentified protein (not depicted).

membrane protein expressed in the endoplasmic reticulum. The bottom panel of Fig. 2 shows a blot with strong staining of total membranes. There was a much weaker signal seen in the eluates, and this signal was comparable in the eluates from kidneys perfused with or without biotin. This therefore represents a small nonspecific binding of the protein to the neutravidin beads, rather than biotinylation of the intracellular protein.

As positive controls for the technique, we assayed the eluates for three Na transport proteins expressed in the luminal membrane of different parts of the nephron. These transporters, although subject to regulation, are thought to be present in the membrane and active under most if not all conditions. Fig. 3 A illustrates detection of the thiazide-sensitive Na-Cl cotransporter (NCC) that is expressed in the distal convoluted tubule (Gamba, 2005). In total membrane proteins the anti-NCC antibody recognizes two proteins with apparent molecular masses of ∼130 and 260 kD. The bottom band is consistent with the monomeric form of the protein, while the top band presumably represents dimers. Previous studies have documented the existence of such dimers that persist under denaturing conditions (de Jong et al., 2003). We did not see either band in blots of kidney medulla, consistent with the presumed distribution of the protein in cortical nephron segments (unpublished data). In the eluate, the putative dimer was the major form of the protein in the eluate while very little of the monomeric form was observed. No protein was detected in eluates from nonbiotinylated kidneys.

We also investigated the bumetanide-sensitive Na/K/2Cl cotransporter NKCC2, which is expressed on the luminal membrane of the thick ascending limb of Henle's loop (Gamba, 2005). In total membranes again both a

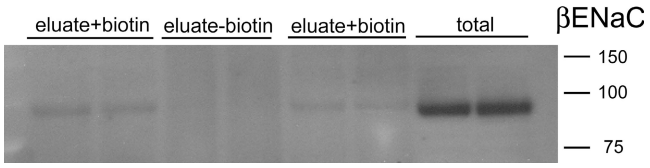

Figure 5. Biotinylation of βENaC. Kidneys from rats on a low-Na diet were perfused with or without biotin. Lanes were loaded with 40 μg of total membrane protein or 20 μl of eluate from the neutravidin beads. Total membranes from whole kidney had a predominant stained band at ~90 kD. Fainter staining at the same molecular mass was observed in eluates from biotinylated kidneys. No staining was detected in eluates from nonbiotinylated kidneys.

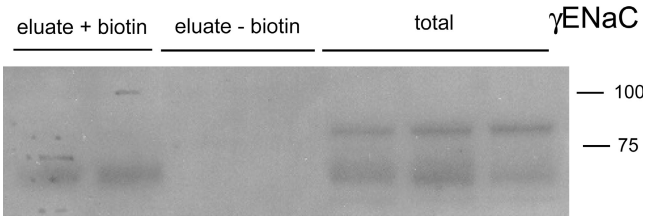

Figure 6. Biotinylation of γENaC. Kidneys from rats on a low-Na diet were perfused with or without biotin. Lanes were loaded with 40 μg of total membrane protein or 20 μl of eluate from the neutravidin beads. Total membranes from whole kidney had two predominant stained bands at ~85 and ~65 kD. Staining of the lower but not the higher molecular mass band was observed in eluates from biotinylated kidneys. No staining was detected in eluates from nonbiotinylated kidneys.

monomeric and a larger, possibly dimeric, form of the protein were present (Fig. 3 B). Previous studies provided evidence for dimerization of the related transporter NKCC1, although in this case the dimers were dissociated in the presence of SDS (Moore-Hoon and Turner, 2000). Higher order multimers of NKCC2 were also detected in rat urine but not in the kidney itself (McKee et al., 2000). As with the NCC, the higher molecular mass species was the major form in the eluate, and the recovery was specific for the biotinylated kidneys.

Finally, we measured the labeling of the Na/H exchanger NHE3, which is present in the luminal membranes of the proximal tubule (Orlowski and Grinstein, 1997). In total kidney membrane fractions the antibody recognized a predominant band of 75 kD (Fig. 3 C). This protein, whose size is consistent with that of a monomeric transporter, was also detected in the eluates of biotinylated but not of nonbiotinylated kidneys. An additional band of approximately twice the size of the major one was also observed in this fraction. It could also represent a dimer of the exchanger protein but we know of no precedent for this. In conclusion, we were able to biotinylate three transport proteins known to be constitutively active in the luminal membranes of different nephron segments. The biotinylated fractions were qualitatively distinct from the total membrane pool in having a greatly enriched presence of the putative dimeric forms of the transporters.

We examined the expression of αENaC in total kidney membranes from rats maintained on a low-Na diet and in biotin/neutravidin eluates as shown in Fig. 4 A. As described previously (Ergonul et al., 2006), the anti-αENaC antibody recognizes three bands. The one with the highest apparent molecular mass (85 kD) corresponds to the full-length form of the subunit. The small fragment that migrates at around 30 kD is thought to reflect proteolysis of the subunit in the N-terminal portion of extracellular domain (Ergonul et al., 2006). The origin of the intermediate band at ~65 kD is not known. Only the small fragment shows up in the eluate, and it is present in roughly equal amounts in the biotinylated and nonbiotinylated tissue. This therefore mainly represents nonspecific interactions between the peptide and the neutravidin-coated beads. However, we believe that the absence of the full-length subunit from the eluate is significant. Biotinylation experiments with *Xenopus* oocytes expressing ENaC indicate that the probe can interact with the 85-kD peptide, as shown in Fig. 4 B. Similar results have been reported previously (Bruns et al., 2007; Harris et al., 2007). Our interpretation is that the absence of this band from the eluate of kidney tissue reflects the absence of the full-length form of the subunit in the apical membrane. If the subunit exists mainly in a cleaved form, then only the 30-kD fragment would be detectable with our antibody directed against the N terminus of the protein. We cannot rule out surface expression of full-length subunit that is undetected due to limited sensitivity of the method.

The surface expression of βENaC from the same rats is shown in Fig. 5. The antibody recognizes a predominant band at ~90 kD, although diffuse staining can be seen above the main band and probably represents subunits with mature glycosylation (Ergonul et al., 2006). The same pattern is present in the eluate, although the amount of protein present is considerably less. The signal was weaker in the eluate, even though 40 μg of total membrane protein was loaded per lane, while the eluate contained material from ~750 μg of total membranes. This indicates that a small fraction of the total protein was recovered from the neutravidin beads. This could indicate that most of the βENaC is intracellular and inaccessible to the biotin probe. However we do not know the efficiency of biotinylation of the plasma membrane proteins, which can be significantly less than 100% (Gottardi et al., 1995). No staining of eluate from nonbiotinylated kidneys was detected, showing that the signal is specific for biotinylated proteins.

A similar blot for γENaC is illustrated in Fig. 6. As in previous studies (Masilamani et al., 1999; Ergonul et al., 2006), the total pool of membranes contains peptides of apparent molecular masses 80 and 65 kD. Under these conditions (animals on a low-Na diet) the two species

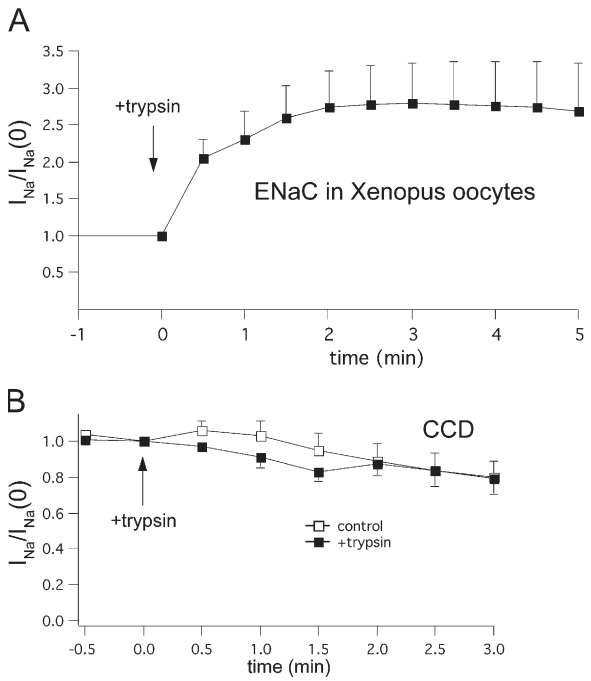

Figure 7. Effects of trypsin on Na^+ channel activity. Current–voltage relationships were generated under voltage-clamp conditions at 30-s intervals. Trypsin (3 μg/ml) was added to the bath at $t = 0$. Currents at −100 mV were corrected for leak current measured in the presence of 10 μM amiloride and normalized to values at $t = 0$. (A) *Xenopus* oocytes. Oocytes expressing rat αβγENaC were preincubated in low-Na solution and superfused with 110 mM Na solution. Currents were measured by two-electrode voltage clamp. Data represent means ± SEM for five oocytes. (B) Rat CCD. Whole-cell currents were measured in principal cells from the CCDs of rats maintained on a low-Na diet for 1 wk. Currents for trypsin treated (filled squares) and time controls (open squares) are plotted. Data represent means ± SEM for 13–14 cells.

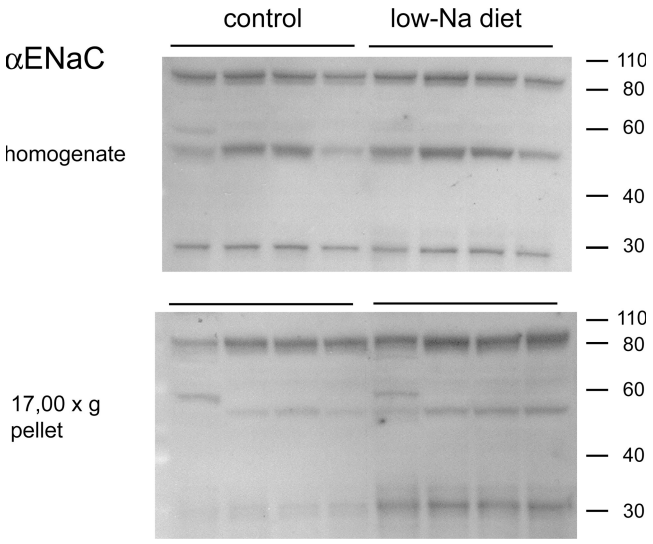

Figure 8. Effect of low-Na diet on αENaC expression in the homogenate and in the intermediate 17,000 *g* membrane pellet of the kidney enriched in surface membranes. Each lane represents 60 μg protein from an individual animal. The expression of the 30-kD fragment was consistently increased by Na depletion, particularly in the intermediate pellet.

were present in roughly equal amounts. The top band corresponds to the full-length subunit, while the bottom band again reflects a proteolytic fragment resulting from cleavage in the N-terminal portion of the extracellular domain. In the eluate, only the cleaved form of the subunit could be detected under these conditions. As for αENaC, it is possible that levels of full-length γENaC below our detection limits were expressed at the surface. Again, only a fraction of the original material was recovered from the neutravidin beads. No signal was observed in the eluate from nonbiotinylated kidneys.

In heterologous expression systems, epithelial Na channels are activated by either intrinsic proteases such as prostasin or furin, or by externally applied proteases such as trypsin or neutraphil elastase (Rossier, 2004; Hughey et al., 2007). Our results suggest that both α and γENaC are present mainly in cleaved forms in the apical membranes of the kidneys of Na-depleted rats. This predicts that native channels should not be strongly activated by trypsin. This prediction is tested as shown in Fig. 7. Panel A shows that addition of the enzyme at a concentration of 3 μg/ml to *Xenopus* oocytes expressing all three ENaC subunits increased amiloride-sensitive current (I_{Na}) by ∼2.5-fold within 2 min. This response is similar to that reported in previous studies in oocytes (Vallet et al., 1997; Hughey et al., 2004). Trypsin from the same stock and at the same concentration failed to increase I_{Na} in cortical collecting ducts (CCDs) isolated from rats maintained on a low-Na diet for 1 wk (Fig. 7 B). Although we cannot rule out a small activation by the protease (see below), most of the channels in the membrane on these tubules appears to be in a trypsin-insensitive form, consistent with the idea that they have previously been cleaved by endogenous proteases. Longer exposures to trypsin led to a gradual inhibition of I_{Na} (unpublished data).

We next investigated the effects of challenge with a low-Na diet on surface expression of ENaC subunits. Since the biotinylation approach did not give useful information on the amount of αENaC at the surface, we used a simpler approach of differential centrifugation to partially purify plasma membranes from intracellular membranes in renal tissue. Marples et al. (1995) showed that the pellet of a 30-min centrifugation at 17,000 *g* was enriched in plasma membranes but not intracellular membranes. In particular, the water channel AQP2 resides in intracellular vesicles in the absence of ADH but moves to the apical membrane in the presence of the hormone. The 17,000 *g* pellet had little AQP2 under low ADH conditions but gained this protein after stimulation, suggesting that this fraction contains apical membranes from the collecting duct principal cells. In addition, we measured the activity of K-dependent phosphatase (Na/K-ATPase), a marker for basolateral plasma

TABLE I

Enzyme Activities in Subcellular Fractions of Rat Kidney

	Homogenate	Intermediate pellet	Final pellet
Na/K-ATPase	20 ± 4	70 ± 6	3 ± 2
NADPH-CytC reductase	15 ± 11	11 ± 9	87 ± 3

Enzyme activities for Na/K-ATPase (K-dependent phosphatase) and NADPH-dependent cytochrome C reductase were measured as described in the text. Data are presented in arbitrary units and represent means ± SEM for eight independent samples.

membranes, and the activity of NADPH-dependent cytochrome C reductase, a marker for endoplasmic reticulum, in subcellular membrane fractions from the kidney. As shown in Table I, the intermediate 17,000 *g* pellet was enriched in the former but depleted of the latter enzyme, while the final pellet was highly enriched in the ER but not the plasma membrane. Thus this fraction contains partially purified apical and basolateral plasma membranes.

As shown in Fig. 8, the low-Na diet produced a significant increase in the abundance of the 30-kD fragment of αENaC in this fraction. In contrast to the biotinylated preparation, the pellet contained full-length αENaC, probably reflecting the lower degree of purity of this fraction compared with that obtained by the biotinylation technique. Similar blots for βENaC and γENaC are shown in Figs. S1 and S2 (available at http://www.jgp.org/cgi/content/full/jgp.200809989/DC1), respectively. For both of these subunits, there was a modest enrichment in the 17,000 *g* pellets, Na depletion produced qualitative changes in the subunits in this pool, with more higher molecular mass (presumably glycosylated) βENaC and more cleaved γENaC. However full-length γENaC was present, again suggesting contamination with intracellular membranes.

Changes in βENaC surface abundance were assessed using biotinylation, as shown in Fig. 9. There was a significant increase in the full-length subunit and a more pronounced accumulation of more slowly migrating protein, suggesting larger amounts of fully glycosylated subunits after Na depletion. The increase in the abundance of the predominant 85-kD band was 1.9-fold.

The changes in γENaC are indicated in Fig. 10. As in Fig. 6, with Na-depleted rats, virtually all of this subunit in the biotinylated fraction was in the low molecular mass (65 kD) form, and this was much more abundant than in the control animals. In controls, much less protein was present in the surface fraction and consisted of approximately equal amounts of intact and cleaved γENaC. The overall increase in surface γENaC estimated from the sum of densities of the larger and smaller band was 3.4-fold, while the increase in the cleaved form was 8.6-fold.

Based on these findings, we repeated the trypsin challenge experiments on CCDs from Na-replete rats. In this case because of the very low channel activity, we used a simpler protocol, measuring whole-cell I_{Na} in different cells in the presence and absence of trypsin. As shown in Fig. 11 A, a small but measurable effect of amiloride on whole-cell currents was detected after treatment with trypsin. This was observed in ~50% of the cells examined. In contrast, none of the cells studied in the absence of trypsin had a measurable I_{Na}. In most cases, the detection limit was ~10 pA/cell or less. Mean values for I_{Na} are shown in Fig. 11 B.

Two additional in vivo conditions were examined that strongly affect channel activity measured in vitro. First, animals were infused for 6–8 d with aldosterone via osmotic minipumps. This leads to channel activities similar to those observed with a low-Na diet and provides comparable, although somewhat lower, steady-state

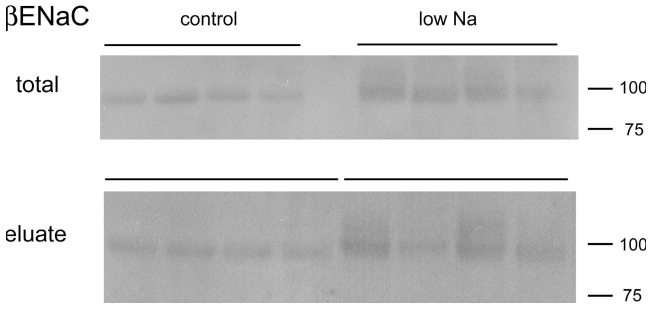

Figure 9. Effect of low-Na diet on βENaC expression in total membranes and in biotin-neutravidin eluates. Each lane was loaded with 75 μg total membrane protein or 25 μl eluate. The expression of the major 90-kD peptide was consistently increased by Na depletion. The staining of more slowly migrating protein, presumably corresponding to glycosylated peptides, was also enhanced.

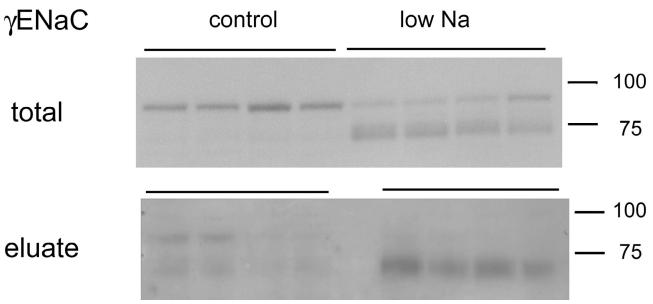

Figure 10. Effects of low-Na diet on γENaC expression in total membranes and in biotin-neutravidin eluates. Each lane was loaded with 75 μg total membrane protein or 25 μl eluate. The staining of low molecular mass peptide was increased, while that of the higher molecular mass peptide was decreased by Na depletion.

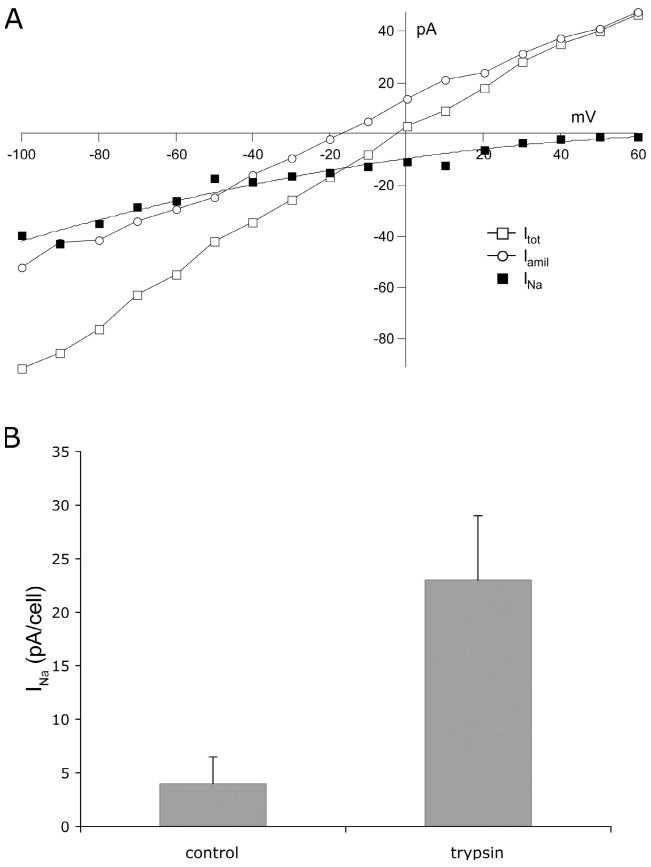

Figure 11. Effect of trypsin on Na-channel activity in CCD from Na-replete rats. (A) Whole-cell current–voltage relationships of a principal cell from a CCD from a rat maintained on control chow. The tubule was superfused with solution containing 3 μg/ml trypsin. Currents were obtained in the absence (open squares) and presence (open circles) of amiloride. Filled squares represent the amiloride-sensitive current (I_{Na}). (B) I_{Na} measured at −100 mV in the presence and absence of trypsin. Data represent means ± SEM for 14 and 13 cells, respectively.

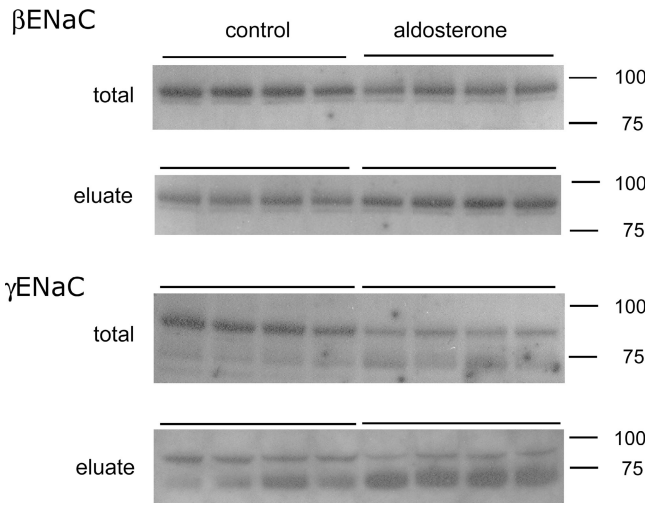

Figure 12. Effects of aldosterone infusion on β and γENaC expression in total membranes and in biotin-neutravidin eluates. Each lane was loaded with 45 μg total membrane protein or 20 μl eluate. Top: staining of the major band of βENaC was slightly decreased in the total membranes but increased in the surface pool of aldosterone-treated animals. Bottom: staining of the lower molecular mass form of γENaC was increased in both the total membranes and in the surface pool.

plasma hormone concentrations (Pácha et al., 1993). Fig. 12 indicates that the amounts of βENaC and of the smaller form of γENaC in the biotin/neutravidin eluates increased similarly to what was seen with Na depletion. A minor difference was that the full-length form of γENaC seemed to persist after aldosterone treatment, although it was much less abundant than the cleaved form. In another set of experiments, animals were fed a low-Na diet for 7 d and then given free access to control diet and saline drinking water for 5 h. We previously showed that this maneuver decreased the whole-cell I_{Na} in CCDs isolated from the animals by 80% (Ergonul et al., 2006). The effects on ENaC subunits in the eluates are shown in Fig. 13. Na repletion decreased the amount of full-length βENaC and cleaved γENaC subunits significantly over this time period.

Fig. 14 summarizes the semiquantitative analysis of the immunoblots. The results for β and γENaC were obtained using biotinylation and are more reliable than those for αENaC. Na depletion increased the amounts of all three subunits at the surface by two- to fourfold. The effect appears to be proportionately greater for γ than for βENaC. However, the increase in βENaC may be underestimated since we did not take into account the high molecular mass, presumably highly glycosylated material. The largest change was in the cleaved form of γENaC, which increased 8–10-fold. This may reflect a combination of an increase in the overall amount of the protein plus a conversion of full-length to cleaved forms. A similar pattern was obtained for the aldosterone-treated vs. control group. Here however the changes in total β and γENaC amounts were comparable. Finally, in the acutely Na-repleted vs. Na-depleted group there was a consistent decrease in surface expression of all three subunits by ∼60%.

DISCUSSION

Biotinylation using membrane-impermeant probes has been used extensively to assess surface expression of membrane proteins (Sargiacomo et al., 1989; Gottardi et al., 1995). Studies of surface expression of epithelial cell membrane proteins have mainly used cultured cell lines in which access to either apical or basolateral cell membranes can be well controlled. In one case, freshly isolated renal tubules were used to measure the surface expression of the Na/K/2Cl cotransporter (Ortiz, 2006). We show here the feasibility of using the entire kidney for such assays. There are no prohibitive technical barriers in this application, which offers several advantages.

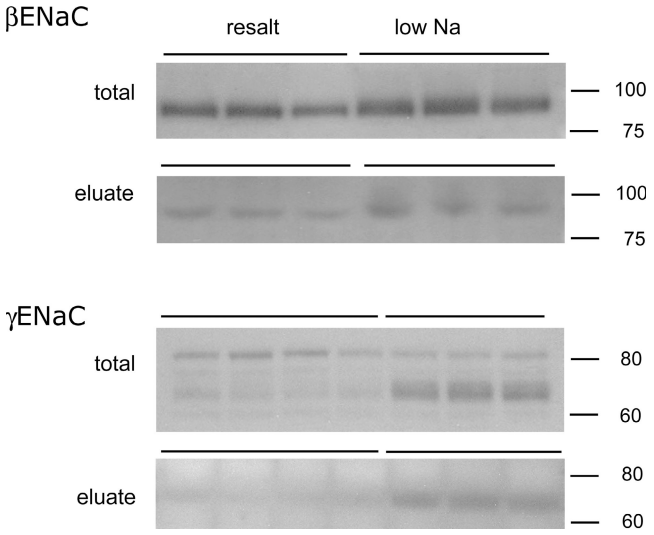

Figure 13. Effect of acute salt repletion on β and γENaC expression in total membranes and in biotin-neutravidin eluates. Each lane was loaded with 40 μg total membrane protein or 20 μl eluate. Staining of the major band of βENaC (top) and the low-molecular mass peptide of γENaC (bottom) were decreased after 5 h salt repletion.

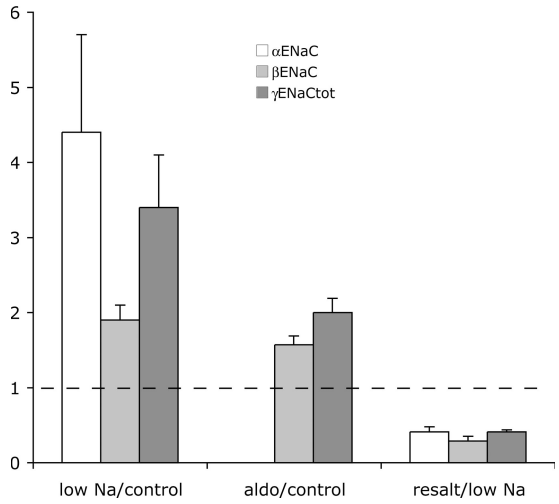

Figure 14. Summary of effects of low Na diet, aldosterone infusion, and acute Na repletion on surface expression of ENaC. Data are plotted as ratios of densities for two conditions and represent means ± SEM for four to eight measurements under each pair of conditions. The αENaC subunit was not analyzed for the aldosterone vs. control conditions. All ratios were significantly different from one ($P < 0.05$).

First, the approach uses native tissue and therefore avoids alterations that may occur in cells that have been grown in culture. Second, the tissue is intact and not subjected to mechanical and/or chemical stresses involved in isolation of cells or nephron fragments. Third, a large amount of tissue is biotinylated. A single kidney from a rat can be used for a large number of assays. Fourth, the entire nephron is biotinylated, permitting the examination of the responses of a number of different segments or transport systems to the same physiological stimulus.

There are also disadvantages and limitations to this approach. For one, a large volume of solution, typically ~200 ml per experiment, is required to perfuse the kidney with the biotinylating reagents. The cost of these reagents can be an issue. In addition we are limited by the availability of antibodies against native proteins. For example, an antibody directed against the C terminus of αENaC might have enabled us to study this subunit using the biotinylation approach. Finally, we could not label apical and basolateral membranes selectively.

Thus far we have used the technique only to study slow changes in kidney function that take place over hours or days. It is not yet clear if it can be applied to more rapid effects. Since such responses also tend to be rapidly reversible it would be necessary to cool the tissue and begin perfusion quickly to obtain a snapshot of the state of the surface expression of membrane proteins. Finally, we have until now only applied the approach to the rat. Adaptation to the mouse should in principle be possible but the surgical techniques would need to be miniaturized and would be more difficult.

Our results are generally similar to those of a previous investigation (Alvarez de la Rosa et al., 2002). That study reported approximately three- to fivefold increases in the surface expression of ENaC subunits in A6 cells from amphibian kidney after 6 h of aldosterone treatment. There are significant differences, however, between the two systems. In A6 cells the three ENaC subunits also increase in overall abundance by three- to fivefold, reflecting similar increases in their rates of translation and smaller, slower changes in rates of transcription (May et al., 1997; Alvarez de la Rosa et al., 2002). In the rat kidney, only αENaC protein increases in abundance in response to both Na depletion and aldosterone treatment (Masilamani et al., 1999; Ergonul et al., 2006), and these increases are modest. Thus the increases in surface expression are at least to some extent dissociated from increased protein synthesis.

Our results are in qualitative agreement with immunocytochemical data (Masilamani et al., 1999; Loffing et al., 2000, 2001; Hager et al., 2001). In those studies, β and γENaC protein was observed to migrate from a diffuse cytoplasmic distribution to a more apical pattern after elevation of plasma aldosterone. The antibody staining pattern was observed at the light microscopic level and it was not possible to discriminate between protein actually in the apical membrane from that which was near the membrane.

The first major conclusion from this work is that γENaC and probably also αENaC subunits are present in the apical membrane mainly in proteolytically cleaved forms, particularly under conditions of high aldosterone. This was shown directly for γENaC and inferred for

αENaC on the basis of the absence of the full-length form of the subunit. Previous work showed that the overall amounts of the cleaved forms correlated with the physiological activity of the channels (Masilamani et al., 1999; Frindt et al., 2001; Ergonul et al., 2006). The cleaved form of γENaC appeared at the expense of the full-length form, while that of αENaC increased in parallel with that of the full-length form. In heterologous expression systems, these subunits were shown to be cleaved by proteases that are plasma membrane–anchored proteases such as CAP1-3 (prostasin), secreted, for instance neutraphil elastase, or Golgi associated including furin (Vallet et al., 1997; Vuagniaux et al., 2002; Hughey et al., 2004; Caldwell et al., 2005; Harris et al., 2007). Furthermore there is strong evidence in these systems that cleavage activates the channels (Caldwell et al., 2004, 2005; Hughey et al., 2004; Bruns et al., 2007; Harris et al., 2007). Our results do not reveal the subcellular site of cleavage in vivo or the enzyme responsible but they do suggest that the channels are mostly cleaved either before they reach the plasma membrane or soon after. However there is evidence for the presence of some full-length γENaC at the surface under Na-replete conditions that disappears during Na depletion (Fig. 10).

The issue of aldosterone-dependent increases in the number of channels at the surface as opposed to hormone activation of surface-expressed channels has been long debated. Early work based on selective modification of surface proteins suggested that aldosterone could affect channels preexisting in the apical membrane (Palmer and Edelman, 1981; Garty and Edelman, 1983). Hormone-dependent increases in channel open probability in A6 cells (Kemendy et al., 1992) were consistent with this idea, although comparable changes were not observed in renal tubules (Pácha et al., 1993). More recent work has suggested aldosterone-dependent alteration in membrane trafficking (Debonneville et al., 2001; Snyder et al., 2002). According to this idea, the aldosterone-induced protein SGK1 inhibits channel ubiquitylation and retrieval by suppressing its interaction with the ubiquitin ligase Nedd4-2.

Our results suggest that trafficking of ENaC to the cell surface accounts for some but not all of the effects of aldosterone. Channel activity, measured as amiloride-sensitive current, is extremely low in the CCD of Na-replete animals (Pácha et al., 1993; Frindt et al., 2001). We estimated that these currents are <10 pA/cell at an electrical driving force of −100 mV (Frindt et al., 2001) compared with very large currents in tubules from Na-depleted or aldosterone-treated rats that can reach as high as 500 pA/cell in the CCD (Frindt et al., 2001) and 1,500 pA/cell in the CNT (Frindt and Palmer, 2004). In contrast, we find measurable expression of ENaC protein at the surface under all conditions, even when currents are undetectably small (Figs. 9, 10, and 12). Put another way, Na depletion or aldosterone treatment produced two- to fivefold increases in surface expression but >50-fold increases in channel activity. This is consistent with activation of channels in the membrane.

This activation could in part result from increased cleavage of the γENaC subunit, as noted above. Indeed, the largest increases in surface expression, up to ninefold, were measured for the 65-kD form of γENaC (Fig. 14). Even this change, however, was smaller than that of the overall channel activity. This suggests that other activating pathways may also help to regulate this transport pathway.

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