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Abstract

An inverse relationship exists between urinary tissue kallikrein (TK) excretion and blood pressure in humans and rodents. In the kidney TK is synthesized in large amounts in the connecting tubule and is mainly released into the urinary fluid where its function remains unknown. In the present study mice with no functional gene coding for TK (TK-/-) were used to test whether the enzyme regulates apically expressed sodium transporters. Semiquantitative immunoblotting of the renal cortex revealed an absence of the 70-kDa form of gamma-ENaC in TK-/- mice. Urinary Na+ excretion after amiloride injection was blunted in TK-/- mice, consistent with reduced renal ENaC activity. Amiloride-sensitive transepithelial potential difference in the colon, where TK is also expressed, was decreased in TK-/- mice, whereas amiloride-sensitive alveolar fluid clearance in the lung, where TK is not expressed, was unchanged. In mice lacking the B2 receptor for kinins, the abundance of the 70-kDa form of gamma-ENaC was increased, indicating that its absence in TK-/- mice is not kinin-mediated. Incubation of membrane proteins from renal cortex of TK-/- mice with TK resulted in the appearance of the 70-kDa band of the gamma-ENaC, indicating that TK was able to promote gamma-ENaC cleavage in vitro. Finally, in mouse cortical collecting ducts isolated and microperfused in vitro, the addition of TK in the luminal fluid increased significantly intracellular Na+ concentration, consistent with an activation of the luminal entry of the cation. The results demonstrate that TK, like several other proteases, can activate ENaC in the kidney and the colon.
DEFECTIVE ENaC PROCESSING AND FUNCTION IN TISSUE-KALLIKREIN DEFICIENT MICE
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Running Head: Proteolytic activation of ENaC by kallikrein

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An inverse relationship exists between urinary tissue kallikrein (TK) excretion and blood pressure in humans and rodents. In the kidney, TK is synthesized in large amounts in the connecting tubule and is mainly released into the urinary fluid where its function remains unknown. In the present study, mice, with no functional gene coding for TK (TK<sup>−/−</sup>) were used to test whether the enzyme regulates apically expressed sodium transporters. Semiquantitative immunoblotting of the renal cortex revealed an absence of the 70 kDa form of γ-ENaC in TK<sup>−/−</sup> mice. Urinary Na<sup+</sup> excretion following amiloride injection was blunted in TK<sup>−/−</sup> mice, consistent with reduced renal ENaC activity. Amiloride-sensitive transepithelial potential difference in the colon, where TK is also expressed, was decreased in TK<sup>−/−</sup> mice whereas amiloride-sensitive alveolar fluid clearance in the lung, where TK is not expressed, was unchanged. In mice lacking the B2 receptor for kinins, the abundance of the 70 kDa form of γ-ENaC was increased, indicating that its absence in TK<sup>−/−</sup> mice is not kinin-mediated. Incubation of membrane proteins from renal cortex of TK<sup>−/−</sup> mice with TK resulted in the appearance of the 70 kDa band of the γ-ENaC, indicating that TK was able to promote γ-ENaC cleavage in vitro. Finally, in mouse cortical collecting ducts isolated and microperfused in vitro, addition of TK in the luminal fluid increased significantly intracellular Na<sup+</sup> concentration, consistent with an activation of the luminal entry of the cation. The results demonstrate that TK, like several other proteases can activate ENaC in the kidney and the colon.

Tissue kallikrein* (TK) is a serine protease that generates kinins locally in many organs, including the kidney, colon and arteries. In the kidney, TK which is synthesized in large amounts by connecting tubule cells (1) is mainly secreted into the urinary fluid and to a lesser extent to the peritubular interstitium. In the renal interstitium, it cleaves locally produced kininogen to yield bradykinin that in turn can activate type-2 (B2) bradykinin receptors. Bradykinin-dependent activation of B2 receptor increases sodium excretion by inhibiting sodium reabsorption in the collecting duct (2). Therefore, the renal kallikrein-kinin system is expected to play a role in renal NaCl balance and blood pressure regulation. Patients with essential hypertension have lower kallikrein levels in their urine (3,4) and mutant mice lacking B2 receptor also exhibit salt-sensitive hypertension (5). However, inactivation of the TK gene in the mouse does not alter blood pressure (6) even though the decrease in renal and urinary kallikrein activity in TK-deficient mice reproduces the phenotype that has been repeatedly associated with hypertension in human and rat studies (7-9). This finding suggests that low urinary kallikrein excretion observed in hypertensive patients is not a
primary cause of high blood pressure (HBP) but rather a consequence of hypertension, or of HBP-associated renal defects. An alternative explanation is that TK-deficient mice develop compensatory mechanisms to keep blood pressure at normal levels.

The role of the large amount of TK that is secreted into the urinary fluid remains unknown. One possibility would be that this protease acts directly on the different transporters expressed at the apical side of the tubular cells to modulate their activities. Apical sodium reabsorption along the renal tubule is achieved through multiple sodium transporters. Particularly, in the aldosterone-sensitive distal nephron (ASDN, i.e., distal convoluted tubule (DCT2), connecting tubule (CNT) and collecting duct), the amiloride-sensitive epithelial Na⁺ channel ENaC, consisting of α-, β-, and γ-subunits, mediates Na⁺ uptake across the apical plasma membrane of principal cells and connecting tubule cells (10). In all renal tubule cells, the Na⁺ is extruded on the basolateral side in exchange for K⁺ by the Na⁺,K⁺-ATPase. Although sodium transport occurs throughout the length of the renal tubule, the fine regulation of sodium excretion occurs in the ASDN, mostly through aldosterone-dependent regulation of ENaC.

As TK production localizes to CNT cells, the urinary side of connecting and collecting duct cells, where ENaC is expressed, is exposed to large amounts of the active enzyme. Because a novel mechanism of proteolytic activation of ENaC by locally produced serine protease has been recently proposed (11,12), we hypothesized that TK might be a paracrine regulator acting directly on ENaC within the ASDN. To test this hypothesis, we used a mouse model with TK gene disruption (TK⁻/⁻) to study molecular and functional expression of ENaC.

**EXPERIMENTAL PROCEDURES**

**Animals.** The TK⁻/⁻ mice were previously generated in our laboratory (6). Type 2-bradykinin receptor knock out mice (B2⁻/⁻) were obtained from the Jackson Laboratory (Bar Harbor, Maine) (13). In all experiments, controls consisted in wild type littermates (WT). All the experimental procedures were performed in accordance with the French Government animal welfare policy (Agreement number RA024647151FR).

**Aldosterone infusion study.** 7 TK⁻/⁻ and 7 WT mice were infused continuously with aldosterone 100 μg.kg body wt⁻¹.day⁻¹ diluted in 0.9% NaCl and 5% DMSO administrated by osmotic minipump (Alzet model 2004, Durect corp., Cupertino, CA). In this particular set of experiments standard laboratory diet was supplemented with 3% Na⁺ and 0.4% K⁺. Control mice received vehicle alone. Infusion of aldosterone or vehicle were continued over a 28-days period.

**Physiological studies.** Animals were housed in metabolic cages and were pair-fed. After 3-5 days adaptation, urines were collected daily for electrolyte measurements. Animals were sacrificed with Ketamine and Xylazine (0.1 and 0.01 mg . g body wt⁻¹, respectively). Plasma and urine electrolytes, creatinine, and aldosterone were determined as described (14). Plasma renin concentration (PRC) was determined by RIA of angiotensin I, generated by incubation of the plasma at pH 8.5 in the presence of an excess of rat angiotensinogen (15). Plasma atrial natriuretic peptide (ANP) concentration was measured by radioimmunoassay (Amersham, Arlington Heights, IL).

**Membrane fraction preparation.** At the time of the sacrifice, kidneys were removed and cut into 5-mm slices. The renal cortex was excised under a stereoscopic microscope and placed into ice-cold isolation buffer (250 mM sucrose, 20 mM Tris-Hepes, pH 7.4) containing protease inhibitors in g/ml: 4 aprotinin, 4 leupeptin, 1.5 pepstatin A and 28 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF). Lung were also removed, minced and placed in the same ice-cold isolation buffer. Minced tissues were homogenized in a Dounce homogenizer (pestle A, 5 passes) followed by 10 passes through a Teflon-glass homogenizer rotating at 1000 rpm. The homogenate was centrifuged at 1000 g for 10 min, and the supernatant was centrifuged at 360,000 g for 40 min at 4°C. The pellet was resuspended in isolation buffer. Protein contents were determined using the Bradford protein assay (microBradford, Bio Rad Laboratories, Hercules, CA).

**Exosome preparation.** Urinary exosomes were prepared as previously described in details by Pitsikun et al. (16). Briefly, urines from TK knock-out and WT mice housed in metabolic cages were collected daily in tubes containing a protease inhibitor mixture (in μg/ml: 1 leupeptin, and 100
and sodium azide. A pool of 75-90 ml urine per group were used. Urine samples were extensively vortexed immediately after they thawed. The urines were centrifuged at 17,000 g for 15 min at 4°C to remove whole cells, large membrane fractions and other debris. Supernatants were centrifuged at 200,000 g for 1 hour at 4°C to obtain a low-density membrane pellet. The exosome-associated proteins isolated from the pooled urine samples were suspended in isolation solution (250 mM sucrose, 10 mM ethanolamine, pH 7.6, containing protease inhibitors in μg/ml: 1 leupeptin, and 100 AEBSF). Protein contents were determined using the Bradford protein assay (Bio Rad Laboratories, Hercules, CA). Values were normalized for urine creatinine to ensure adequate protein loading in western blot experiments, i.e., to allow comparison of the abundance in exosome associated proteins from urine samples of the same time for each group, as proposed by others (17).

**Antibodies.** Rabbit polyclonal antibodies to NHE3 and NKCC2 have been characterized previously (19). Rabbit polyclonal antibodies to NaPi-2a was given by J. Biber (Zürich University, Switzerland) (18). Rabbit polyclonal antibodies to NaPi-2a was and NKCC2 have been characterized previously and has been characterized previously (19).

Membrane proteins were solubilized in SDS-loading buffer (62.5 mM Tris HCl, pH 6.8, 2% SDS, 100 mM dithiothreitol, 10% glycerol and bromophenol blue), incubated at room temperature for 30 min. Electrophoresis was initially performed for all samples on 7.5% polyacrylamide minigels (XCell SureLock Mini-cell, Invitrogen Life Technologies), which were stained with Coomasie blue to provide quantitative assessment of loading, as previously described (14). For immunoblotting, proteins were transferred electrophoretically (XCell II Blot Module, Invitrogen Life Technologies) for 1.5 h at 4°C from unstained gels to nitrocellulose membranes (Amersham, Arlington Heights, IL) and then stained with 0.5% Ponceau S in acetic acid to check uniformity of protein transfer onto the nitrocellulose membrane. Membranes were first incubated in 5% nonfat dry milk in phosphate-buffered saline, pH 7.4 (PBS) for 1 h at room temperature to block nonspecific binding of antibody, followed by overnight at 4°C with the primary antibody (anti-NHE3 1:1000; anti-NaPi2 1:20000; anti-NCC 1:5000; anti-NKCC2 1:5000; anti-α-ENaC 1:3000; anti-β-ENaC 1:20000; anti-γ-ENaC 1:2000 ; anti-Na+/K+ATPase 1:20000) in PBS containing 1% nonfat dry milk. After four 5 min washes in PBS containing 0.1% Tween-20, membranes were incubated with 1:10000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA) or peroxidase-conjugated affiniPure donkey anti-chicken IgY (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) in PBS containing 5% nonfat dry milk for 2 hours at room temperature. Blots were washed as above, and luminol-enhanced chemiluminescence (ECL, Perkin Elmer Life Science Products, Inc. Boston, MA) was used to visualize bound antibodies before exposure to Hyperfilm ECL (Amersham, Arlington Heights, IL). The autoradiography was digitized with use of a laser scanner (Epson Perfection 1650, Epson), and quantification of each band was performed by densitometry using NIH Image software. Densitometric values were normalized to the mean for the control group that was defined as 100% and results were expressed as mean ± S.E.

For deglycosylation experiments, protein samples were incubated for 1 hour at 37 °C with or without peptide N-glycosidase F (PNGase F) according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany), and processed for immunoblotting as described above.

To assess whether or not TK promotes γ-ENaC cleavage in vitro, renal cortical membrane fractions from TK−/− mice, were prepared as described above except that proteases inhibitors were omitted. To compare their ability to promote γ-ENaC cleavage, urines from TK−/− and WT mice were first desalted against Tris buffer (0.2 M, pH 8.2) in a centricron column (Centricon-10, cut-off 10 kDa, Millipore). Final volume of the desalted urine was 1/3 of the initial volume of urine introduced in
the column. Membrane fractions were incubated with desalted urines for 1.5 h at 37°C, or with purified TK from porcine pancreas (Sigma-Aldrich, Lyon, France) before SDS-PAGE and immunoblotting with anti γ-ENaC antibody as described above.

**Measurement of alveolar fluid clearance in mouse.** Sodium-driven alveolar fluid clearance (AFC) was measured in vivo using an in situ non-ventilated mouse lung model, as previously described (22,23). This model has been shown to give AFC values similar to those obtained with the ventilated mouse model over a 15-min period. Briefly, male or female wild type or TK−/− mice aged 2-5 months were euthanized with intraperitoneal pentobarbital (250 mg/kg) and maintained at 37-38°C using a heating pad, an infrared lamp and an intraabdominal monitoring thermistor. A 20-gauge venous catheter was inserted in the trachea through a tracheotomy and tightly fixed. The lungs were inflated with 100% O2 at 7 cm H2O continuous positive airway pressure throughout the experiment. Then, 10 ml/kg of instillate was delivered to the lungs over 30 s through the tracheal catheter. The instillate consisted of Ringer’s lactate (pH 7.4) adjusted to 325 mosmol/kg H2O with NaCl, containing 5% bovine serum albumin (BSA) and 0.1 µCi/ml 125I-albumin (Cis Bio International, Gif-sur-Yvette, France) as a labelled alveolar fluid volume tracer. An alveolar fluid sample (50-100 µl) was aspirated 1 min after instillation and at the end of experiment (15 min later). The aspirates were centrifuged at 3,000 g for 10 min, and the radioactivity in supernatants was counted in duplicate. Alveolar fluid clearance (percentage fluid absorption at 15 min) was calculated from the increase in alveolar fluid albumin as follows : AFC (%) = (Cf – Ci) / Cf x 100, where Ci and Cf represent the initial and final concentrations of 125I-albumin in the aspirate at 1 and 15 min respectively, as assessed by radioactivity measurements. In some experiments, amiloride (1 mM) was added to the instillate and AFC was measured at 15 min as described above. ENaC-mediated AFC represents the difference between total AFC values (in the absence of amiloride) and amiloride-insensitive AFC values (in the presence of amiloride). Results are presented as means ± SE. One-way variance analyses were performed and, when allowed by the F value, results were compared by the modified least significant difference (Statview software). P<0.05 was considered significant.

**Measurement of rectal transepithelial potential difference.** The mice were anesthetized with an intra-peritoneal injection of Ketalar (Park-Davis, Courbevoie, France) 75µg/g body weight and Rompun (Bayer, Puteaux, France) 2.3µg/g body weight and placed on a heated table. A winged needle filled with isotonic saline was placed in the subcutaneous tissue of the back. A double-barreled pipette was prepared from borosilicate glass capillaries (1.0 mm OD/0.5 mm ID, Hilgerberg, Malsfeld, Germany) and pulled to a ~0.2 mm tip diameter. The first barrel was filled with isotonic saline buffered with 10 mM Na+-HEPES (pH 7.2), and the second barrel with the same solution containing 25 µM amiloride. The tip of the double-barreled pipette was placed in the rectum about 3-5 mm from the skin margin. The electrical potential difference was measured between the first barrel and the subcutaneous needle, both connected to Ag/AgCl electrodes by means of plastic tubes filled with 3 M KCl in 2% agar. The rectal PD was monitored continuously by a VCC600 electrometer (Physiologic Instruments, San Diego, CA) connected to a chart-paper recorder. After stabilization of the rectal PD (about 1 min), 0.05 ml of saline solution was injected through the first barrel as a control maneuver and the PD was recorded for another 30-second period. A similar volume of saline solution containing 25 µM amiloride was injected through the second barrel of the pipette and the PD was recorded for another 1 minute. The amiloride-sensitive PD was calculated as the difference between the PD recorded before and after addition of amiloride.

**Evaluation of apical Na⁺ entry in isolated CCD microperfused in vitro.** Experiments were performed as previously described by others (24). Briefly, CCD segments were dissected from C57Bl/6 mouse, mounted on concentric pipettes and perfused in vitro. During intracellular pH measurement experiments, the average tubule length exposed to bath fluid was limited to 300 – 350 µm in order to prevent motion of the tubule. The dissection and initial luminal and peritubular solutions were composed of (in mM) 25 NaCl, 119 Na-methyl-D-glucamine gluconate, 1.2 MgSO4, 2 K2HPO4, 2 CaCl2, 5 D-glucose, and 10 HEPES and was adjusted to a pH of 7.4. Tubules were bathed and perfused with this same solution. For
the experiments, CCDs were bathed in a modified solution in which NaCl was isosmotically replaced with N-methyl-D-glucamine gluconate to achieve an NaCl concentration of 0 mmol/L and containing the Na ionophore monensin (10^-5 M). This maneuver effectively eliminated the basolateral membrane as a barrier for Na movement. Under these conditions, Na entry across the apical membrane is the only means of altering [Na]i.

To identify principal and intercalated cells, we labeled intercalated cells by adding fluorescein-labeled peanut lectin (PNA, Vector Labs) to the luminal perfusate for 5 minutes and observed which cells were fluorescent with excitation and emission wavelengths of 440 and 530 nm, respectively. [Na]i in CCD cells was assessed with imaging-based, dual excitation-wavelength fluorescence microscopy with use of the fluorescent probe sodium-binding benzofuran isophthalate (SBFI, Molecular probes). Tubules were loaded with 2x10^-5 M of the acetoxy methyl ester of SBFI added to the luminal perfusate. Intracellular dye was excited alternatively at 340 and 380 nm with a 100-watt Xenon lamp and a computer-controlled chopper assembly. Emitted light was collected through a dichroïc mirror, passed through a 510 nm filter and focused onto a CCD camera (ICCD 2525F, Videoscope International, VA) connected to a computer. The measured light intensities were digitized with 8-bit precision (256 grey level scale) for further analysis. For each tubule, 2 to 4 principal cells were analyzed: the mean grey level for each excitation wavelength was calculated with the Starwise Fluo software (Imstar, Paris, France). SBFI fluorescence ratios (340/380 nm) were used as an estimator of [Na], values.

**RESULTS**

Evidence for decreased sodium channel activity in the kidney and the distal colon. TK is secreted into the luminal fluid of the distal part of the ASDN, where ENaC is believed to be the limiting step for Na⁺ reabsorption, and an strong inverse relationship has been described between the amount of TK excreted in the urine and blood pressure in humans (3,4). We speculated that TK might be a factor regulating ENaC activity. To test renal ENaC function in vivo, we first tested the effects of amiloride on urinary sodium excretion in TK⁻/⁻ mice. Fig 1A shows that a single dose of amiloride (subcutaneous injection of 1.45 mg . kg bw⁻¹) significantly increased urinary Na⁺ excretion during the next two hours following the injection in WT mice whereas the effect of amiloride on Na⁺ excretion was blunted in TK⁻/⁻ mice. This result, indicated that renal ENaC activity was decreased in mice lacking TK production.

The distal colon also expresses all three ENaC subunits, mRNA for TK has been found to be highly expressed in the colon, and abundant TK activity is measurable in the feces (6). Thus, we hypothesized that if TK activates ENaC, this effect may be detectable in the distal colon. We tested this hypothesis in vivo by measuring amiloride-sensitive transepithelial rectal potential difference, an index of ENaC-mediated Na⁺ transport, in WT and TK⁻/⁻ mice. As shown in Figure 1B, the amiloride-sensitive rectal potential difference was significantly decreased in TK⁻/⁻ mice compared to WT mice, indicating that as in the kidney, TK is an activating factor of ENaC in the distal colon.

Table 1 shows that TK⁻/⁻ mice were able to maintain normal Na⁺ balance with no evidence for extracellular fluid volume depletion. Moreover, the animals were pair fed, and hence identical urinary excretion of Na⁺ and Cl⁻ (see Table 1) indicated that they were able to achieve a steady state. Thus, although the TK⁻/⁻ mice are able to excrete the full amount of NaCl taken in the diet and maintain a steady state, they do so with a concomitant decrease in amiloride-sensitive (ENaC-mediated) sodium absorption, implying that amiloride-independent Na⁺ absorption, presumably upstream from the collecting duct system, is increased.

Luminal TK activates ENaC in principal cells of cortical collecting duct isolated and microperfused in vitro. Our preceding experiments indicate that TK⁻/⁻ mice have a decreased in ENaC-mediated Na⁺ transport in kidney. Therefore, to confirm more directly that TK is able to activate ENaC, we next examined the effects of extracellular (luminal) TK on ENaC in CCDs isolated and microperfused in vitro. Intercalated cells were identified by adding fluorescein-labeled peanut lectin to the luminal perfusate for 5 minutes and observed which cells were fluorescent. [Na]i in principal cells was then assessed with use of the fluorescent probe sodium-binding benzofuran isophthalate (SBFI), as previously described (24). ENaC is the limiting step
for Na entry into principal cells, therefore, if TK activates ENaC, [Na], is expected to increase when TK is perfused to the luminal surface of the CCD. Accordingly, Figure 2 shows that TK was able to increase significantly the ratio of F340/F380 SBFI fluorescence, which reflects an increase in [Na⁺], whereas no change was observed in CCD perfused with vehicle only. Moreover, TK-dependent increase in [Na⁺], was prevented when the tubules were pre-treated with 10⁻⁵M amiloride, indicating that the effect of TK on [Na], is a consequence of ENaC activation.

γENaC processing is impaired in TK⁻/⁻ mice. Because TK is a serine protease, it is possible that TK-induced ENaC activation occurs through ENaC proteolysis. Thus, we next examined ENaC-related polypeptides by western blot. As shown in Figure 3, antibodies to the C-terminus of γ-ENaC detect a major band at 85 kDa, as well as a minor 80 kDa band. An additional broad band centered around 70 kDa was detected in WT mice but not in TK⁻/⁻ mice. This 70 kDa band is believed to originate from the proteolytic cleavage of the N-terminal end of the 85 kDa form and to be related to aldosterone-dependent activation of ENaC (20). When renal samples from WT mice were deglycosylated with PNGase and immunoblotted using anti-γENaC, the 85-kDa band shifted to 72 kDa and the broad 70 kDa band shifted to a major 50-kDa band and a weaker 60-kDa band (Fig 4A). No change in protein abundance of either the α- and β-subunits of ENaC nor other Na⁺ transporter expressed along the nephron was detected (Fig 3, Table 2).

TK is able to promote the cleavage of γ-ENaC in vitro. If TK is important for the activation of ENaC via its proteolytic properties on γ-ENaC subunit, TK should be able to promote the cleavage of this protein in vitro. Renal membrane fractions from TK⁻/⁻ mice, prepared in the absence of protease inhibitors to avoid in vitro inactivation of TK, were incubated with porcine pancreatic TK at 100 or 30 μg/ml, or with vehicle alone for 15 minutes at 37 °C. Figure 5A shows that only the 85 kDa band is detected on renal membrane fractions from TK⁻/⁻ mice exposed to the vehicle and that the 70 kDa band of the γ-ENaC appeared in samples incubated with both concentrations of TK. Densitometric analyses were performed to quantify the appearance of the kallikrein induced 70 kDa γ-ENaC (Figure 5B). Band densities of the 70 kDa band of 4 independent immunoblots were normalized to total densities of the γ-ENaC. The 70 kDa band was significantly increased by TK at both concentrations over control. At the highest concentration of TK, the total densities of γ-ENaC was significantly decreased presumably due to nonspecific protein degradation.

Porcine pancreatic TK is extracted from pancreas and might therefore contain small amount of other protease, particularly trypsin, which is known to activate ENaC. Thus, in a second set of experiments, desalted urines collected from WT and TK⁻/⁻ mice and renal membrane fraction from TK⁻/⁻ mice (i.e. naturally devoid of detectable 70 kDa form of γ-ENaC, see Fig. 3) were used. As shown in Figure 5C, when renal membrane fractions prepared from a TK⁻/⁻ mouse were incubated with desalted urines from either WT or TK⁻/⁻ mice, the 70 kDa band of γ-ENaC appeared in samples incubated with urine from WT mice but not with urine from TK⁻/⁻ mice, indicating that urinary kallikrein favors cleavage of γ-ENaC in vitro. No cleaved band appeared in untreated samples kept in parallel over the period of the experiment indicating that the 70 kDa band is not the result of nonspecific degradation.

Impaired γENaC processing leads to decreased abundance in urinary exosomes. Study of urinary exosome-associated proteins provides a means for analysis of changes in apical protein expression of renal tubular cells (16). We looked at changes in the excretion of the three subunits of ENaC in three independent preparations of urinary exosomes from WT and TK⁻/⁻ mice by immunoblotting using anti-N terminus α-ENaC antibody, and anti-C terminus β- or γ-ENaC antibodies. Figure 6 shows representative immunoblots. Only the β- and γ-subunits of ENaC were detectable in the urinary exosome preparations from WT mice. In human exosome preparations, all three subunits were detectable in urinary exosomes, suggesting that this is the only molecular form of γ-ENaC present at the apical surface of the collecting duct cells. As expected from the results shown in Figure 3, the γ-subunit of
ENaC was dramatically decreased in urinary exosome preparation from TK+/+ mice (25 ± 2 % of control, p<0.001). We also observed a marked decrease in β-ENaC protein abundance (24 ± 6 % of control, p<0.01). As in WT mice, α-ENaC was undetectable in urinary exosomes from TK-/- mice. Importantly, the protein abundance of NCC, assessed on same preparations, was not altered (102 ± 34 % of control, p=0.96), indicating that the decrease in β- and γ-ENaC subunits is specific and not due to incomplete sampling of urine.

**Semiquantitative immunoblots for γ-ENaC in mutant mice lacking kinin-B2 receptor (B2-/-).** To rule out the possibility that the disappearance of the renal 70 kDa form of γ-ENaC in TK-/- mice might be the consequence of the absence of locally-generated bradykinin rather than a direct effect of TK disruption on ENaC proteolysis, we next performed immunoblots for γ-ENaC on cortical membrane fractions obtained from WT and B2-/- mice (Figure 7). The combined densities of the 85 kDa and 70 kDa bands were unchanged in B2-/- mice (100 ± 3 % vs. 100% ± 6 % in TK-/- and WT mice, respectively). In contrast to what was observed in TK-/- mice (see Figure 1A), the abundance of the 70 kDa form of γ-ENaC was increased in B2-/- mice (159 ± 23 % vs. 100 ± 6 % in B2-/- and WT mice, respectively, p = 0.03). There was no significant difference in the abundance of the 85 kDa form of γ-ENaC (84 ± 13 % compared with wild type, 100 ± 6%).

**Evidence for unchanged sodium channel activity in lung.** In contrast to the kidney and the colon, lung alveolar epithelial cells express ENaC but do not secrete TK, therefore we hypothesized that ENaC processing in the lung is TK-independent. Fig 8A shows that amiloride-sensitive alveolar fluid clearance, used as an estimate of ENaC-mediated Na+ transport (23), was not significantly different in TK-/- mice compared with WT mice. Figure 8B shows that the biochemical profile of γ-ENaC in the lung appears as two major bands, one at 85 kDa similar to that one observed in the kidney and another one resolving at 75-70 kDa. These bands were no longer detected when anti γ-ENaC was preincubated with the peptide antigen (data not shown). There was no significant difference in the abundance of the 85 kDa γ-ENaC (102 ± 5 % vs. 100 ± 12%, in TK-/- and WT mice respectively) and of the 75-70 kDa γ-ENaC (103 ± 6 % vs. 100 ± 8%, in TK-/- and WT mice respectively). Deglycosylation with PNGase gave a pattern similar to that one seen in renal samples from WT mice except that the size of the major fragment was 60 kDa (data not shown).

**Adaptation of TK deficient mice to dietary Na+ restriction and response to aldosterone infusion**

As stated above, TK-/- mice showed no evidence for altered blood volume. However, it is not unusual that altered sodium transport in transgenic mice leads to limited phenotypes. Despite decreased ENaC activity in the absence of TK production, TK-/- were able to adapt normally in response to acute dietary sodium restriction. Both genotypes significantly decreased urinary Na+ excretion after Na+ restriction. No significant difference were observed among groups (Data not shown). In addition, urinary aldosterone levels increased similarly in TK-/- (11.96 ± 3.35 nmol/mmol creatinine) and WT mice (12.21 ± 1.64 nmol/mmol creatinine) after 3 days Na+ depletion. This result indicated that in response to Na+ restriction, TK-/- mice were able restore normal ENaC function. In WT animals undergoing sodium restriction, the broad 70 kDa band was easily detected on immunoblots for γ-ENaC (Fig. 9A), consistent with previous observations (20,25,26). In TK-/- mice, sodium depletion was associated with the appearance of polypeptides of molecular weights ranging from 75 to 70 kDa (compare WT and TK-/- lanes in Fig. 7A). Same results were observed in mice treated with aldosterone (Fig 9B). Deglycosylation with PNGase of aldosterone infused WT and TK-/- mice gave a similar pattern except that the size of the major fragments was 50 kDa and 60 kDa for WT and TK-/- mice, respectively (Fig. 4B).These sets of experiments demonstrate that in the nominal absence of TK ENaC can still be processed and activated by other serine proteases, presumably from the CAP family.

**DISCUSSION**

The present study demonstrates that TK is a physiological activator of ENaC. This activation is associated with the proteolytic cleavage of the full length 85 kDa form of γ-ENaC subunit within its
extracellular domain to produce a truncated peptide of 70 kDa corresponding to the C-terminal end of the entire protein. This demonstration is based on several observations: 1) TK-/- mice have decreased ENaC activity in the kidney and also the distal colon, two organs that exhibit ENaC-mediated Na+ transport and abundantly produce TK; 2) TK increased ENaC-dependent Na+ entry when applied to the luminal surface of CCDs isolated and microperfused in vitro; 3) At steady state, TK deficient mice have a marked decrease in the protein abundance of the truncated γ-ENaC peptide in the kidney and in urinary exosomes, a preparation that reflects the pool of protein apically expressed in renal tubular cells; 4) TK is able to promote the cleavage of γ-ENaC in vitro; 5) In the lung alveolae, an organ devoid of TK expression, TK disruption did not impair ENaC processing and activity.

ENaC is a hetero-multimeric protein composed of three related subunits α, β and γ. All subunits have an intracytoplasmic N- and C-terminus, and two membrane spanning domains connected by a large extracellular loop. ENaC activity can be regulated by changes in membrane protein abundance of the channel or by changes in single-channel open probability. Recently, it has been proposed that proteolytic processing of α- and γ- subunits by several serine proteases increases ENaC open probability (11,12,27-29). Conversely, non-processed ENaC have been demonstrated to be almost inactive when expressed in cell cultures. In this model, extracellular trypsin stimulates ENaC, whereas extracellular serine protease inhibitors, such as aprotinin and bikunin, have been shown to decrease ENaC activity (12,29-32). However, despite this evidence that proteolytic processing of ENaC stimulates channel activity, the molecular identity of the protease that could mediate this effect in vivo is still uncertain. As stated above, trypsin can activate ENaC. Elastase, a serine protease naturally produced by neutrophil polynuclear has also been proposed to activate ENaC in vivo during pulmonary inflammatory states (28). More recently, direct evidence for exogenous cleavage of γ-ENaC and activation at the cell surface by elastase in Xenopus oocytes experiments has been provided by Harris et al. (33). However, trypsin and elastase are not very good candidates for in vivo physiological regulation of ENaC in the kidney because they are normally not expressed and secreted in this organ. Furin, a proprotein convertase involved in many physiological or pathophysiological processes, including activation of hormones and growth factors to bacterial toxins and viral glycoproteins processing is also able to cleave and activate ENaC in vitro (11,34). A family of serine proteases has been identified that could cleave and activate ENaC, including CAP1, CAP2, CAP3, and TMPRSS3 (12,23,35-38). Importantly, it has been shown, that aldosterone modifies urinary excretion of prostasin, the human ortholog of CAP1 (39), suggesting that prostasin could be the link between aldosterone and proteolytic activation of ENaC in the kidney. Unfortunately, gene disruption of furin or CAP1 are lethal rendering difficult direct assessment of their physiological role in ENaC regulation (40,41). It is also possible that proteolytic activation of ENaC involves more than one effector. This is supported by the observation that the proteolytic processing of γ-ENaC by furin seems to occur intracellularly, whereas trypsin, elastase or the CAPs are believed to act extracellularly. Moreover, it has recently been demonstrated that furin or prostasin are able to produce different polypeptides from γ-ENaC (42).

Here we report that a serine protease promotes γ-ENaC cleavage in vivo and in vitro (see figure 1A and 5) and that this proteolytic modification correlates with the activation of ENaC. Active TK excretion is positively correlated with mineralocorticoid levels (43,44). Interestingly, it has also been shown that the protein abundance of the 70 kDa form of γ-ENaC (the truncated form which disappears in TK-/- mice) also correlates with circulating aldosterone level (20). Therefore, it is tempting to conclude that TK is responsible for part of the aldosterone-dependent processing/activation of the channel. However, it is important to state that it is not possible to conclude that TK is directly responsible for the cleavage itself (i.e. that TK physically interacts with and cleaves γ-ENaC). Indeed, in our in vitro experiments we used membrane fractions from TK-/- mice. This material does not contain TK but it probably expresses several other proteases, particularly several members of the CAP family that are membrane-bound. Therefore, it is possible that the action of TK on γ-ENaC is indirect. For example, it is
conceivable that TK activates a CAP-dependent signaling cascade leading ultimately to ENaC processing as proposed by B. Rossier recently (45).

Since the TK−/− mice did not exhibit gross alterations in sodium balance or extracellular fluid regulation, it could be also argued that TK does not play a substantial physiological role under normal circumstances. However, since regulation of Na− reabsorption in the kidney is critical for extracellular fluid volume and blood pressure regulation, the mechanisms for regulation of Na− reabsorption are highly highly redundant. That is, animals can generally compensate for defective regulation at one renal tubule segment by increasing absorption at another renal tubule segment, lowering blood pressure or reducing GFR to come into Na− balance. Thus, in the present study, altered regulation at the level of ENaC is likely to be compensated for. One example of this is seen in mice in which the thiazide-sensitive NaCl cotransporter, responsible for 5-10 percent of sodium reabsorption under normal circumstance (46). These mice appear healthy and are normal with respect to plasma electrolyte concentrations, serum aldosterone levels, and blood pressure. Moreover, NCC−/− mice retain Na− as well as wild-type mice when fed a Na−-depleted diet (47). In contrast, these mice show abnormalities in homeostasis of other ions including Ca++ (47) and K+ (48). One other possible explanation is that the TK gene inactivation does not only disrupt TK production but also impairs local bradykinin production. Inhibition of bradykinin production is expected to limit natriuresis while TK disruption is expected to limit ENaC activation. Therefore, the phenotype arising from both effects might be very limited. Finally, in the absence of TK, some proteolytic activation of the channel can still occur and produce several different polypeptides that are presumably active (see Fig 9). The role of furin or CAPs in this compensation requires further investigation. For example, CAP could permit the cleavage of the 85-kDa γ-ENaC form and therefore activation of ENaC in TK−/− mice with elevated circulating aldosterone. Based on the size of the fragments obtained following deglycosylation of the cleaved form of γ-ENaC (60 and 50 kDa) which are recognized by the anti γ-ENaC antibody directed against the COOH terminus of γ-ENaC, the proteolytic cleavage seems to occur at two different sites in the early portion of the extracellular loop of the γ-ENaC, which are approximately one hundred amino acid apart. Deglycosylation of renal samples from either control, or aldosterone treated WT mice showed that the 70 kDa protein shifted to a major 50 kDa protein (figure 2). Deglycosylation of kidney samples from aldosterone infused TK−/− mice showed that the broad 75-70 kDa protein shifted to a major 60-kDa protein and a weaker 50-kDa protein (figure 2). Membrane samples from lung, an organ not expressing TK (6) showed a similar pattern following deglycosylation. These results suggest that the renal 70-kDa γ-ENaC could result from the proteolytic cleavage of the 85-kDa γ-ENaC form by the TK itself (or at least dependent on TK), whereas the 75-70 kDa broad protein seen in kidney sample from TK−/− mice with elevated circulating aldosterone or lung sample from either WT or TK−/− mice could result from the proteolytic cleavage of the 85-kDa γ-ENaC by another serine protease such as CAPs or furin. We also report that ENaC is functionally normal in lung alveoli of TK−/− mice, confirming that ENaC in the lung can be processed by another protease than TK. These observations highlight the fact that TK-dependent activation of ENaC may be tissue specific.

In conclusion, this study identified an antinatriuretic effect of TK, and we propose that TK is a physiological activator of ENaC that belongs to a complex cascade of protease that are required for full stimulation of Na transport within the ASDN. The data from our model of TK inactivation strongly suggest that the decrease in renal TK production that has been found repeatedly to be associated with high blood pressure in humans is rather a consequence than a cause of this disease.

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**FOOTNOTES**

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*In this paper, Tissue Kallikrein (TK) indicates the product of the mouse *klk1* gene (accession number : NM_010639), synonyms : KLK1, Kal, mGk-6, renal kallikrein, Klk1b6."
**FIGURE LEGENDS**

Figure 1: Effects of TK disruption on ENaC function in the kidney and colon. A) Effect of amiloride injection on urinary Na$^+$ excretion in TK$^{-/-}$ and WT mice. Mice kept under normal Na$^+$ diet (0.3 % Na$^+$ in food) were subcutaneously injected with amiloride (1.45 mg/kg body weight). Urines from TK$^{-/-}$ (filled square) and WT mice (filled triangle) were collected over two days from 9 to 11 am, 11 am to 2 pm, 2 to 6 pm and 6 pm to 9 am; amiloride was injected at 9 am the second day. UNa$^+$/Ucreatinine represents the urinary Na$^+$ concentration in mM normalized to the urinary concentration of creatinine in mM. Values are the means ± SE from 5 to 9 determinations in each group. *P<0.05, **P<0.01 vs time controls; # P<0.01 vs TK$^{-/-}$ mice. B) Diurnal variations in amiloride-sensitive rectal potential difference (PD) in TK knockout mice. In vivo measurements of amiloride-sensitive rectal potential difference (PD) in TK knockout mice. In vivo measurements of amiloride-sensitive rectal potential difference (PD) in TK$^{-/-}$ (white bars) and WT (black bars) mice were performed in the morning (10~12 am) and afternoon (4~6 pm). The data show a significant decrease in amiloride-sensitive sodium transport in the colon of TK knock-out mice (n= 4 to 6 mice per group). *P <0.05.

Figure 2: Time course of SBFI fluorescence, an estimate of [Na$^+$], in principal cells of CCDs isolated and microperfused in vitro. CCDs isolated from WT mice were perfused with either TK (10μg/ml) in the absence (filled circles) or the presence of 10$^{-5}$M amiloride (asterisk), or perfused with vehicle alone (open circles). After 10 min of TK perfusion, SBFI fluorescence increased significantly in TK treated tubules (p<0.05 vs. controls), reflecting a rise in [Na$^+$], whereas no change occurred in CCD perfused with vehicle. Moreover, 10$^{-5}$M amiloride in the luminal fluid prevented the TK-induced rise in [Na$^+$], demonstrating that this effect reflects ENaC activation by TK. (n=3 in each group)

Figure 3: Immunoblot of renal cortex membrane fractions from TK$^{-/-}$ and WT mice with antibodies to α-, β-, and γ-subunit of ENaC. The 70kDa γ-ENaC polypeptides were undetectable, whereas α-, and β-subunits-related polypeptides were unchanged. Each lane was loaded with a protein sample from a different mouse. Equal loading was confirmed by parallel Coomassie stained gels.

Figure 4: Glycosylation pattern of γ-ENaC protein in mouse renal cortex. Protein samples (A, membrane fractions from TK$^{-/-}$ and WT mice under basal conditions and aldosterone infused TK$^{-/-}$ and WT mice renal cortex ; B, membrane fractions from aldosterone infused TK$^{-/-}$ and WT mice renal cortex) were incubated with or without N-glycosydase F (PNGase F) for 1 hour at 37°C before SDS-PAGE and immunoblotting for γ-ENaC

Figure 5: Tissue Kallikrein promotes γ-ENaC cleavage in vitro. Panel A: Renal membrane fractions from TK$^{-/-}$ mice were incubated with TK and apparition of the 70 kDa form of γ-ENaC was studied by immunoblotting. Membrane proteins were mixed with either 0, 30 or 100μg/ml of TK purified from porcine pancreas and incubated for 15 min. at 37°C. Panel B: Densitometric analyses of data showing the appearance of the 70 kDa form following treatment with TK. Panel C: Renal membrane fractions from TK$^{-/-}$ mice were incubated with desalted and concentrated urines from WT or TK$^{-/-}$ mice for 1.5 hours at 37°C. The apparition of the 70 kDa form of γ-ENaC was studied by immunoblotting.

Figure 6: Abundance of ENaC subunits and NCC by immunoblotting in urinary exosomes from TK knockout and WT mice normalized to urine creatinine, and kidney cortex from wild type mouse. Lanes, from left to right, were loaded with protein samples from wild type mouse kidney cortex (lane 1), urinary exosomes from wild type mice (lane 2) and urinary exosomes from TK knockout mice (lane 3). Immunoblots are representative of three independent experiments.
**Figure 7**: Abundance of γ-ENaC protein by immunoblotting in membrane fractions from kidney cortex of $B2^{-/-}$ and WT mice. Panel A: Immunoblot of renal cortex membrane fractions from $TK^{-/-}$ and WT mice. Each lane was loaded with a protein sample from a different mouse. Equal loading was confirmed by parallel Coomassie stained gels. Panel B: Densitometric analyses of immunoblots showing that the abundance of the 70 kDa form is significantly increased in $B2$ receptor knockout mice. *: $P<0.05$ versus WT mice.

**Figure 8**: Analyses of γ-ENaC polypeptides and of ENaC function in the lung. Panel A, Effect of $TK$ knock-out on sodium-driven alveolar fluid clearance in mice. Sodium-driven alveolar fluid clearance (AFC) was measured over a 15 min-period in WT and $TK^{-/-}$ mice aged 2-5 months at 37°C using an *in situ* non ventilated model in which the airspace was instilled with an isoosmolar Ringer’s lactate solution containing $^{125}$I-albumin as a volume marker, as described in Methods. Experiments were performed either in the absence (Total AFC, black bars) or in the presence of 1mM amiloride (Amil, white bars) in the instillate. Results are expressed as percentage fluid absorption at 15 min, and represent means ± SE of 8-9 mice in each group for total AFC experiments, and 4 mice in each group for amiloride experiments. Panel B: Abundance of γ-ENaC protein by immunoblotting in membrane fractions from lung of $TK^{-/-}$ and WT mice. Each lane was loaded with a protein sample from a different mouse. Equal loading was confirmed by parallel Coomassie stained gels. No significant differences in the abundance levels of the 85 kDa band and the 75-70 kDa band were observed between WT and $TK^{-/-}$ mice. *: significantly different from corresponding total AFC value ($P<0.05$). NS: not significantly different.

**Figure 9**: Effect of $TK$ knock-out on γ-ENaC protein in mice undergoing dietary Na$^+$ restriction or chronic aldosterone infusion. Immunoblots for γ-ENaC of membrane fractions from kidney cortex of $TK$ knockout and wild type mice under Na$^+$ restricted diet (A) or following chronic aldosterone administration (B). Each lane was loaded with a protein sample from a different mouse. Equal loading was confirmed by parallel Coomassie stained gels. * indicates a protein sample from a mouse that had not correctly received aldosterone.
Table I. Physiological parameters from WT and \( TK^{-/-} \) mice kept under a \( Na^{+} \)-replete diet. Values are means ± SE. Statistical significance between group was determined by unpaired Student t-test.

|                         | WT (n=7)   | TK\(^{-/-}\) (n=7) | \( p \) |
|-------------------------|------------|---------------------|--------|
| Renin activity          | 868 ± 133  | 531 ± 71.4          | 0.049  |
| (ng AngioI/h/ml)        |            |                     |        |
| Atrial natriuretic peptide (pg/ml) | 325 ± 27 | 361 ± 23          | 0.35   |
| Aldosterone (pM)        | 555 ± 172  | 504 ± 102           | 0.81   |
| Protein (g/l)           | 48.7 ± 0.9 | 47.5 ± 0.6          | 0.32   |
| Vu (mL/24h)             | 1.28 ± 0.61| 1.45 ± 0.25         | 0.60   |
| Creatinine (μmol/day)   | 4.28 ± 1.75| 5.91 ± 1.05         | 0.34   |
| Aldosterone (nmol/mmol Creat) | 1.74 ± 0.20 | 1.82 ± 0.13 | 0.76   |
| Na\(^{+}\) (mmol/mmol Creat) | 23.6 ± 3.5 | 19.2 ± 1.8         | 0.29   |
| K\(^{+}\) (mmol/mmol Creat) | 24.0 ± 3.1 | 19.1 ± 1.2         | 0.19   |
| Cl\(^{-}\) (mmol/mmol Creat) | 40.3 ± 0.9 | 36.5 ± 1.1        | 0.07   |
Table 2: Summary of densitometric analyses of data obtained by western-blot on cortical membrane fractions. Values are means ± SE. Statistical significance between group was determined by unpaired Student t-test.

|          | NaPi-2a | NHE3 | NKCC2 | NCC | α-ENaC | β-ENaC | γ-ENaC 85 kDa | total | Na/K-ATPase α subunit | β subunit |
|----------|---------|------|-------|-----|--------|--------|---------------|-------|---------------------|----------|
| **WT (n=5)** | 100 ± 12 | 100 ± 10 | 100 ± 30 | 100 ± 14 | 100 ± 20 | 100 ± 6 | 100 ± 12 | 100 ± 7 | 100 ± 20 |
| **TK−/− (n=5)** | 93 ± 8 | 108 ± 14 | 100 ± 18 | 80 ± 10 | 86 ± 12 | 96 ± 8 | 122 ± 5 | 98 ± 2 | 80 ± 8 | 97 ± 17 |
| **p**      | N.S.    | N.S.  | N.S.  | N.S. | N.S.   | N.S.   | 0.07          | N.S.  | N.S.    | N.S.     |

p values: N.S. = not significant.
Figure 2

[Graph showing F340/F380 (arbitrary units) vs. Time (min) with data points for Control, Kallikrein, and Kallikrein + Amiloride]
Figure 4

A  Control
WT   TK-/-

B  Aldo
WT   TK-/-

kDa
50 -
60 -
70 -
85 -

PNGase -  +  -  +  -  +  -  +
Figure 5

A

B

C

70 kDa γ-ENaC protein abundance (% of total γ-ENaC)

Kallikrein, µg/ml

WT TK−/− 0 WT TK−/− 0

KDa

85

70

t=0 t=1.5
Figure 7

Panel A shows a gel with molecular weight markers in kilodaltons (kDa) for WT and B2−− samples. Panel B presents a bar graph indicating the abundance of γENaC protein compared to control, with 70 kDa and 85 kDa markers, showing a significant difference (*) between WT and B2−− samples.
Figure 8

A

Alveolar fluid clearance (% fluid cleared / 15min)

WT  

TK⁻/⁻  

NS

B

kDa

85  

75  

70  

WT  

TK⁻/⁻
Figure 9

A

\[
\begin{array}{c}
\text{kDa} \\
85- \\
70- \\
\end{array}
\]

\begin{tabular}{cc}
\text{WT} & \text{TK}^{-/-} \\
\end{tabular}

B

\[
\begin{array}{c}
\text{kDa} \\
85- \\
70- \\
\end{array}
\]

\begin{tabular}{cc}
\text{WT} & \text{TK}^{-/-} \\
\end{tabular}

\text{*}