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Plasmin Activates Epithelial Na^+ Channels by Cleaving the γ Subunit*

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Proteolytic processing of epithelial sodium channel (ENaC) subunits occurs as channels mature within the biosynthetic pathway. The proteolytic processing events of the α and γ subunits are associated with channel activation. Furin cleaves the α subunit ectodomain at two sites, releasing an inhibitory tract and activating the channel. However, furin cleaves the γ subunit ectodomain only once. A second distal cleavage in the γ subunit induced by other proteases, such as prostasin and elastase, is required to release a second inhibitory tract and further activate the channel. We found that the serine protease plasmin activates ENaC in association with inducing cleavage of the γ subunit at γLys[194], a site distal to the furin site. A γK194A mutant prevented both plasmin-dependent activation of ENaC and plasmin-dependent production of a unique 70-kDa carboxyl-terminal γ subunit cleavage fragment. Plasmin-dependent cleavage and activation of ENaC may have a role in extracellular volume expansion in human disorders associated with proteinuria, as filtered plasminogen may be processed by urokinase, released from renal tubular epithelium, to generate active plasmin.

The epithelial sodium channel (ENaC)[3] transports Na^+ across the apical membrane of principal cells in the aldosterone-sensitive distal nephron (1). Alterations in ENaC activity disrupt Na^+ balance, leading to changes in both extracellular volume and blood pressure. Expansion of extracellular volume occurs in a variety of clinical disorders, including nephrotic syndrome. The role of ENaC activation in many of the clinical disorders associated with extracellular volume expansion remains to be defined.

ENaC activity in the cells that line the distal nephron depends on the number of channels in the apical membrane and on channel open probability (P_o) (1). ENaC subunits undergo post-translational processing by specific proteases (2–9). Cleavage of the α and γ subunits by proteases has a key role in activating ENaC, presumably by releasing inhibitory domains within the ectodomains of the α and γ subunits (3, 5, 10, 11). We have proposed that multiple proteolytic cleavage events lead to a stepwise activation of ENaC, reflected in a stepwise increase in channel P_o (3, 5, 10, 12). Channels that lack proteolytic processing have a low P_o (3, 10, 12, 13). Channels that have been cleaved solely by furin, where an α subunit inhibitory tract has been released, exhibit an intermediate P_o (3, 10, 12). Furin-processed channels likely represent the channels that are observed in *Xenopus* oocytes at a single channel level. Channels that have released both α and γ subunit inhibitory tracts exhibit a high P_o, as we observed in oocytes co-expressing ENaC and prostasin (3, 5). Both non-cleaved channels and furin-processed channels at the plasma membrane provide a reservoir of channels that can be activated by extracellular proteases (3, 14).

One potential activator of ENaC is the serine protease plasmin. Although known for its involvement in fibrinolysis, plasmin has been implicated in other processes, including tumor pathogenesis, inflammation, and atherosclerosis (15). Plasmin cleaves after basic residues (Arg and Lys) similar to trypsin, a known activator of ENaC (16, 17). Plasminogen, the inactive precursor to plasmin, is recovered at higher concentrations in the urine of individuals with nephrotic syndrome compared with healthy individuals (18, 19). Urokinase, an enzyme that converts plasminogen to plasmin, is released by cells lining both the proximal and distal nephron (20–22). As active plasmin may be present in the distal nephron in the setting of nephrotic syndrome, it is important to determine whether plasmin can cleave and activate ENaC. We have now shown that extracellular plasmin activates ENaC expressed in *Xenopus* oocytes. Channel activation by plasmin is dependent on cleavage of the γ subunit at a site that is in close proximity to defined sites for prostasin- and elastase-dependent cleavage of the γ subunit (5, 7, 8).

EXPERIMENTAL PROCEDURES

DNA Constructs, Site-directed Mutagenesis, and cRNAs—Wild-type and mutant mouse γENaC constructs, including the prostasin-dependent cleavage site mutant (RKRK[186] to QQQQ[186]), contained amino-terminal HA and carboxyl-terminal V5 epitope tags as described previously (5, 23). The γK194A point mutation was generated using a PCR-based approach (24). T3 or T7 mMessage mMachine (Ambion, Aus-

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[3] The abbreviations used are: ENaC, epithelial sodium channel; P_o, open probability; TEV, two-electrode voltage clamp; HA, hemagglutinin; MDCK, Madin-Darby canine kidney.

tin, TX) was used to synthesize cRNAs for αENaC, βENaC, and γENaC (wild-type and mutant).

Functional Expression in Xenopus Oocytes—ENaC subunits were expressed in Stage V–VI *Xenopus laevis* oocytes pretreated with 1.5 mg/ml type IV collagenase (25). ENaC cRNAs were injected at a concentration of 2 ng/subunit/oocyte. Electrophysiological measurements were performed at 24–32 h post injection using two-electrode voltage clamp (TEV) as described previously (25). The bath solution was 100 mM sodium gluconate, 1.54 mM $CaCl_2$, 5 mM $BaCl_2$, 10 mM HEPES, 10 mM tetraethylammonium chloride, pH 7.4. Selected oocytes were bathed in TEV buffer with bovine plasmin (10 μg/ml; Innovative Research, Inc., Novi, MI) for defined time periods. The amiloride-insensitive component of the whole cell current was determined by perfusion with bath solution supplemented with amiloride (100 μM). Currents measured at −60 mV were recorded at base line and at varying times of exposure to plasmin.

Detection of the Surface Pool of ENaC in Oocytes—Twenty-four h post-injection, oocytes (15–40 per group) were placed in modified Barth's saline (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 15 mM HEPES, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, pH 7.4) supplemented with 10 μM amiloride. Oocytes expressing ENaC containing wild-type or mutant γ subunits were incubated with or without plasmin (10 μg/ml) for 4 min at room temperature and then placed immediately on ice and washed with ice-cold modified Barth's saline. Oocyte surface proteins were biotinylated, and surface V5-tagged ENaC was recovered with streptavidin beads (Pierce) for immunoblotting with anti-V5 antibodies as described previously (5, 7).

Detection of the Surface Pool and Total Cellular Pool of ENaC in Madin-Darby Canine Kidney (MDCK) Cells—Type I MDCK cells from Barry Gumbiner (University of Virginia, Charlottesville, VA), grown on plastic wells, were transiently transfected with epitope-tagged αβγENaC (α-Myc, β-FLAG, and either HA-γ-V5 or HA-γRKRK^{186}/QQQQ-V5) cDNAs using Lipofectamine 2000 (Invitrogen) as described previously (23). Twenty-four h post-transfection, ENaC was immunoprecipitated from cell extracts with anti-V5 antibodies and Protein G conjugated to Sepharose as previously described (23) prior to incubation for 0, 6, and 18 min with 15 μl of purified plasmin (20 μg/ml) in phosphate buffered saline (137 mM NaCl, 2.6 mM KCl, 15.2 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.5 mM $MgCl_2$, and 0.7 mM $CaCl_2$). The beads were washed, and the immunoprecipitate was subjected to immunoblotting with anti-V5 antibodies. Alternatively, MDCK cells expressing ENaC were treated with 1 ml of 3, 9, or 27 μg/ml plasmin in phosphate-buffered saline for 6 min at 37 °C and then treated with EZ-Link sulfo-NHS-SS-biotin (Pierce) to biotinylate surface proteins as described previously (23). Cells were solubilized, and ENaC was immunoprecipitated with anti-V5 antibodies and Protein G conjugated to Sepharose as described previously (23). Proteins from washed beads were eluted with 50 μl of 1% (w/v) SDS in HEPES-buffered saline (150 mM NaCl, 10 mM HEPES, pH 7.4) by heating to 95 °C for 2 min. Samples were diluted with 1 ml of 1% (v/v) Triton X-100 in HEPES-buffered saline, and biotinylated surface ENaC subunits were recovered with 30 μl of streptavidin-conjugated beads (Pierce). The beads were washed, eluted into Laemmli sample buffer (Bio-Rad) containing 10% β-mercaptoethanol and 2% SDS by heating to 95 °C for 3.5 min, and immunoblotted as described previously (23).

Analysis of Rat Urine—Male ZSF1 rats (diabetic and hypertensive (16 weeks old)) and lean littermates (11 weeks old) were placed in metabolic cages, and urine was collected and frozen as described previously (26). Urine protein concentrations were determined using the Bradford assay (Pierce). Urine aliquots of 0.15 ml from five different lean control rats (see Fig. 7, *lanes 1–5*), containing between 0.12–0.32 mg of protein, and obese rats (see *lanes 6–10*), containing between 0.26–0.48 mg of protein, were separately concentrated 10-fold using ProteoSpin™ urine protein concentration kit from Norgen BioTek Corp. (Ontario, Canada). Samples (15 μl) were subjected to SDS-PAGE on a 4–15% gradient gel (Bio-Rad Criterion™ precast gel) under reducing conditions (10% β-mercaptoethanol) and analyzed by immunoblotting with goat anti-human plasminogen antibodies (American Diagnostica, Inc., Greenwich, CT) and rabbit horseradish peroxidase-conjugated secondary antibodies (Rockland, Gilbertsville, PA).

Statistical Analysis—Data are presented as the mean ± S.E. The S.E. for base line normalized currents was calculated by dividing the S.E. of the amiloride-sensitive current by the mean current. Unpaired Student's *t* test was used to determine significance between groups, with $p < 0.05$ considered to be statistically significant.

RESULTS

Plasmin Treatment Increases Amiloride-sensitive ENaC Currents and Cleaves the γ Subunit—We and others have previously shown that ENaC expressed in *Xenopus* oocytes is activated by furin-dependent cleavage of the α and γ subunits. Co-expression of ENaC and prostasin in oocytes further activates the channel by inducing cleavage of the γ subunit at a second site distal to the furin site (5). ENaC expressed in oocytes can also be activated by treating oocytes with extracellular proteases, such as trypsin or elastase (4, 7, 8). Neutrophil elastase also induces cleavage of the γ subunit at a second distal site (7, 8). To determine whether plasmin activates ENaC, whole cell currents in oocytes expressing mouse αβγENaC, with or without treatment with 10 μg/ml plasmin, were measured by TEV (Fig. 1). A time-dependent increase in whole cell amiloride-sensitive current was observed using TEV within 1 min, and the current continued to increase over 6 min (Fig. 1*A*), similar to the time course for activating ENaC by external trypsin (27, 28). The current increase reached a maximum by 10 min and then remained stable for an additional 20 min (Fig. 1*B*). In the absence of plasmin treatment, whole cell currents remained relatively stable. Plasmin did not activate currents in non-injected oocytes (data not shown). Exposure of ENaC-expressing oocytes to plasmin (10 μg/ml) for 4 min significantly increased amiloride-sensitive ENaC currents by 1.95-fold ($p < 0.005$) (Fig. 1*C*).

MDCK cells expressing epitope-tagged αβγENaC (α-Myc, β-FLAG, and HA-γ-V5) were treated with increasing amounts of plasmin (0, 3, 9, and 27 μg/ml) for 6 min to determine

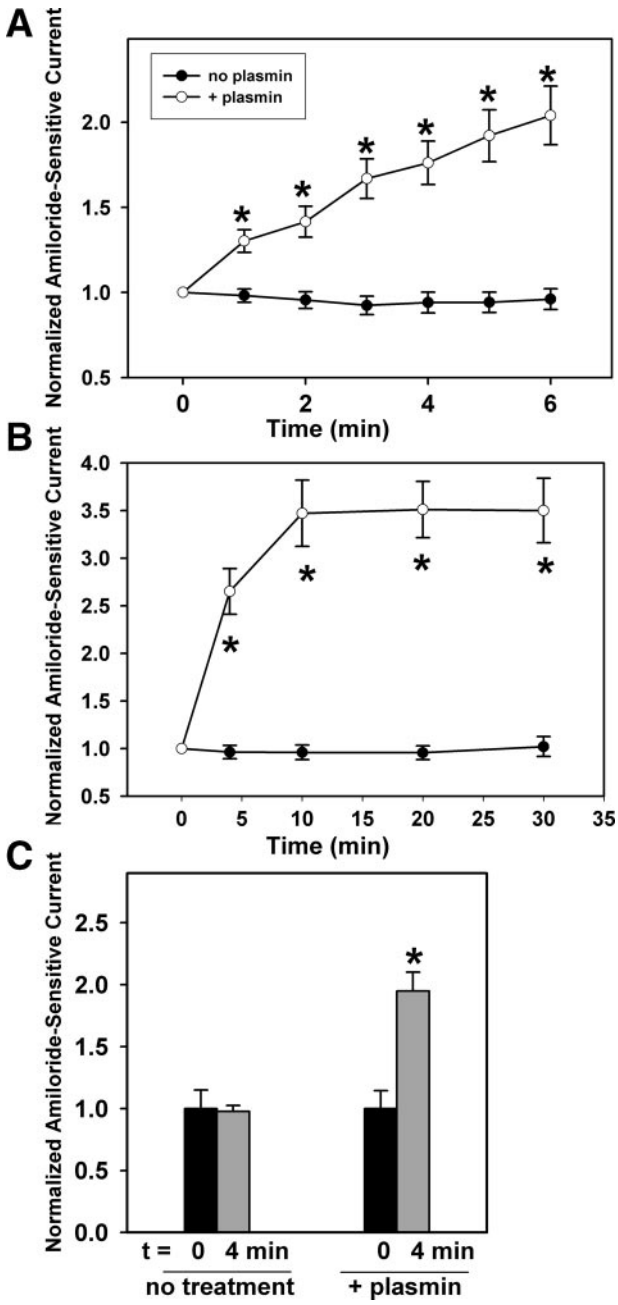

FIGURE 1. **Plasmin activates mouse ENaC expressed in *Xenopus* oocytes.** Oocytes injected with cRNAs for non-tagged α and β and HA-γ-V5 were analyzed by TEV before and after exposure to plasmin (10 μg/ml) for the indicated times. *A* and *B*, time course for activation of amiloride-sensitive Na^+ currents in oocytes treated with external plasmin (10 μg/ml) is shown. *A*, for control oocytes, amiloride-sensitive currents at base line were -2330 ± 390 nA (mean and S.E., $n = 17$). For oocytes exposed to 10 μg/ml plasmin, amiloride-sensitive currents at base line were -2020 ± 300 nA (mean and S.E., $n = 16$–17). Normalized currents are also shown. *, $p < 0.005$, plasmin-treated *versus* control normalized currents at each time point following the initiation of plasmin treatment. *B*, for control oocytes, amiloride-sensitive currents at base line were -1560 ± 310 nA (mean and S.E., $n = 8$–10). For oocytes exposed to 10 μg/ml plasmin, amiloride-sensitive currents at base line were -1320 ± 330 nA (mean and S.E., $n = 11$). Normalized currents are also shown. *, $p < 0.005$, plasmin-treated *versus* control normalized currents at each time point following the initiation of plasmin treatment. *C*, for control oocytes, amiloride-sensitive currents at base line were -1700 ± 260 nA (mean and S.E., $n = 27$). For oocytes exposed to 10 μg/ml plasmin, amiloride-sensitive currents at baseline were -1810 ± 260 nA (mean and S.E., $n = 23$). Normalized currents are also shown. *, $p < 0.005$, plasmin-treated *versus* control normalized currents at each time point following the initiation of plasmin treatment. Treatment of oocytes for 4 min with plasmin (10 μg/ml) increased amiloride-sensitive sodium currents by 1.95-fold.

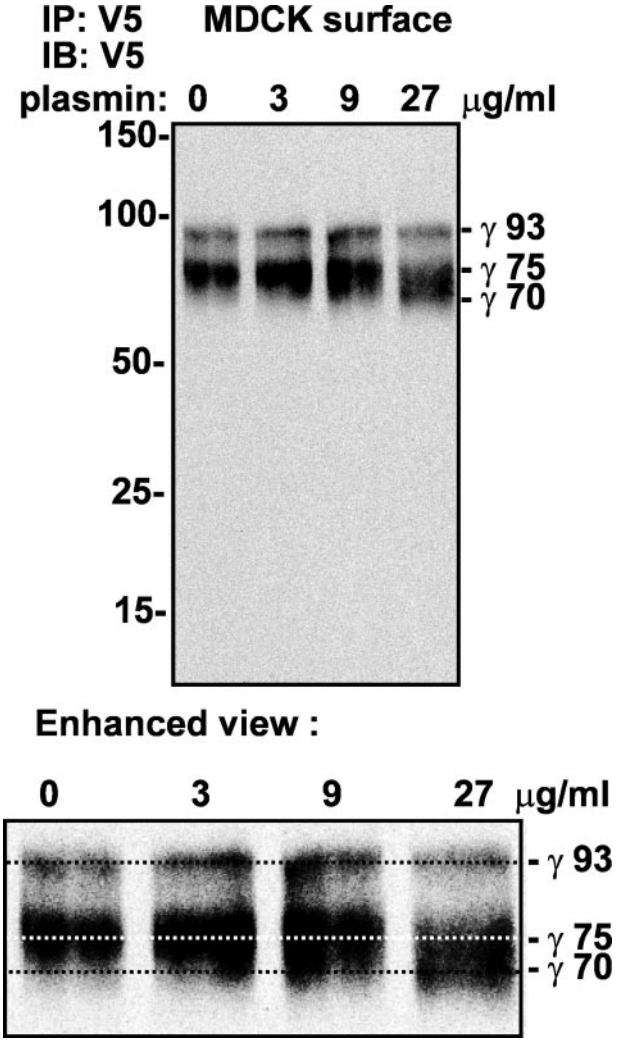

FIGURE 2. **Plasmin cleaves the γ subunit at a site distal to the furin cleavage site when ENaC is expressed in MDCK cells.** MDCK cells were transiently transfected with α-Myc, β-FLAG, and HA-γ-V5. The following day, cells were exposed to 0, 3, 9, or 27 μg/ml plasmin for 6 min prior to biotinylation of surface proteins. Biotinylated γ was recovered with streptavidin-conjugated beads from anti-V5 immunoprecipitates (*IP*) and analyzed by immunoblotting (*IB*) with anti-V5 antibodies. Mobilities of the non-cleaved 93-kDa γ (γ *93*), furin-cleaved 75-kDa γ (γ *75*), and the new plasmin-dependent 70-kDa γ (γ *70*) fragments are indicated to the *right* of the gel. An enhanced version of the banding pattern is shown in the *lower panel* and includes three parallel lines that denote γ93, γ75, and γ70. *Numbers* to the left of the gel represent the mobility of Bio-Rad Precision Plus protein standards in kDa on a 7.5% gel. The blot is representative of three independent experiments.

whether plasmin cleaves γ at a site distinct from that cleaved by furin. After plasmin treatment, surface proteins were biotinylated. Following serial precipitation with anti-V5 antibodies and streptavidin-conjugated beads, immunoblots were probed with anti-V5 antibodies (Fig. 2). Both the non-cleaved (93 kDa) and furin-cleaved (75 kDa) forms of γ were present at the cell surface in the absence of plasmin exposure. However, an additional band at 70 kDa was observed when cells were treated with increasing concentrations of plasmin. The size of the new fragment was consistent with plasmin cleaving γ at a site distal to the furin cleavage site at Arg^{143}, and was similar in size to the prostasin-dependent cleavage fragment resulting from cleavage at $RKRK^{186}$ (5).

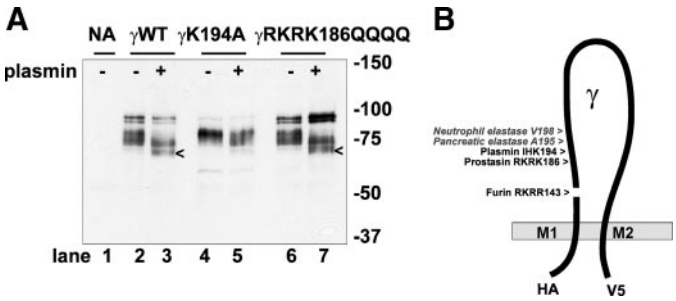

FIGURE 3. **Plasmin cleaves at γLys^{194} when ENaC is expressed in *Xenopus* oocytes.** *A*, *Xenopus* oocytes were injected with cRNA for wild-type α and β and either wild-type γ (*γWT*) or γ with mutation of the prostasin cleavage site (γRKRK^{186}QQQQ) or the plasmin cleavage site predicted at γIHK^{194} (γK194A). *NA*, no addition CRNA. All γ constructs contained N-terminal HA and C-terminal V5 epitope tags. The next day, oocytes were treated with plasmin (10 μg/ml) for 4 min prior to surface biotinylation. Biotinylated proteins were precipitated with streptavidin beads and eluted for analysis by immunoblotting with anti-V5 antibodies. Appearance of the 70-kDa band (<) due to plasmin cleavage is blocked by the γK194A mutation. *B*, model of γENaC denoting the cleavage sites determined for furin (γRKRR^{143}), prostasin (γRKRK^{186}), pancreatic elastase (γAla^{195}), neutrophil elastase (γVal^{198}), and now, plasmin (γIHK^{194}) (4, 5, 7, 8). See "Results" for details. *Numbers* to the right of the gel represent the mobility of Bio-Rad Precision Plus protein standards in kDa on a 10% gel.

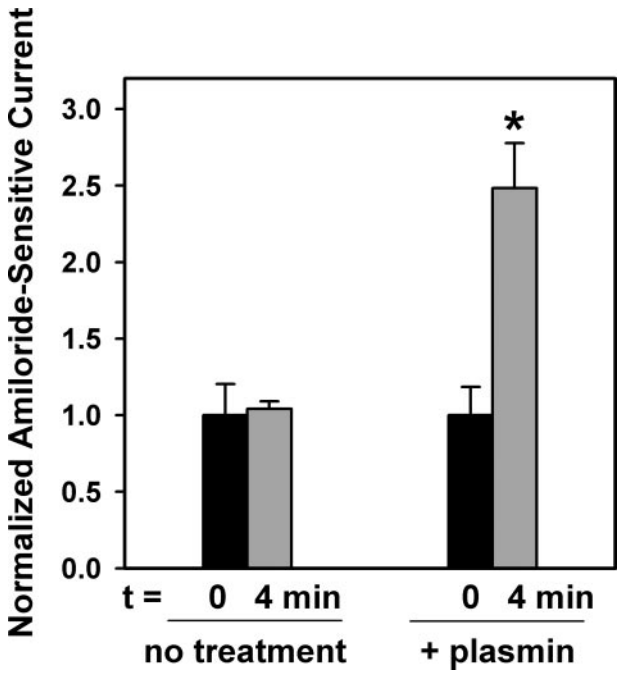

FIGURE 4. **Plasmin activates ENaC containing a γ subunit with a prostasin cleavage site mutation.** Oocytes injected with cRNAs for non-tagged α and β and mutant HA-γRKRK^{186}/QQQQ-V5 were analyzed by TEV before and after exposure to plasmin (10 μg/ml) for 4 min. For control oocytes, amiloride-sensitive base-line currents were −2410 ± 490 nA (mean and S.E., $n = 12$). For oocytes exposed to plasmin, amiloride-sensitive currents at base line were −1740 ± 320 nA (mean and S.E., $n = 15$). Application of extracellular plasmin (10 μg/ml) for 4 min increased mutant ENaC currents by 2.48-fold. *, $p <$ 0.005, plasmin-treated *versus* control normalized currents at 4 min.

Oocytes expressing αβγENaC (α, β, and HA-γ-V5) were also treated with plasmin (0 or 10 μg/ml) for 4 min to determine whether plasmin cleaves γ at a site distinct from that cleaved by furin. After plasmin treatment, surface proteins were biotinylated and precipitated with streptavidin beads, and immunoblots were probed with anti-V5 antibodies. As observed with ENaC expressed in MDCK cells, both the non-cleaved (93 kDa) and furin-cleaved (75 kDa) forms of γ were present at the cell surface in the absence of plasmin treatment. The additional band at 70 kDa was observed when oocytes were exposed to plasmin (Fig. 3*A*, *lanes 2* and *3*).

Mutation of the Prostasin Cleavage Site Does Not Block Plasmin-dependent Activation of ENaC or γ Cleavage—The prostasin consensus site mutation γRKRK^{186}/QQQQ blocks both ENaC activation by prostasin when co-expressed in oocytes and prostasin-dependent γ cleavage when ENaC and prostasin are co-expressed in MDCK cells (5). In contrast, we observed that ENaC containing γRKRK^{186}/QQQQ was significantly activated 2.48-fold in oocytes exposed to 10 μg/ml plasmin for 4 min (Fig. 4). Plasmin cleavage of the γ subunit was also not affected by the γRKRK^{186}/QQQQ mutation (Figs. 3 and 5). ENaC containing either wild-type γ or mutant γRKRK^{186}/QQQQ was immunoprecipitated from transfected MDCK cells and subsequently treated with plasmin (20 μg/ml) for 0, 6, or 18 min. In both cases, immunoblot analysis revealed the non-cleaved (93 kDa) and furin-cleaved (75 kDa) forms of γ in the absence of plasmin ($t = 0$), whereas incubation with plasmin for 6 or 18 min revealed the plasmin-dependent 70-kDa fragment.

Oocytes expressing αβγRKRK^{186}/QQQQ (α, β, and HA-γ-V5) were also treated with plasmin (0 or 10 μg/ml) for 4 min. Following plasmin treatment, surface proteins were biotinylated and precipitated with streptavidin beads, and immunoblots were probed with anti-V5 antibodies. As observed with αβγENaC, an additional 70-kDa band was observed when oocytes expressing αβγRKRK186/QQQQ were exposed to plasmin (Fig. 3*A*, *lanes 6* and *7*).

Plasmin Activation of ENaC and Plasmin Cleavage of γ Are Blocked by the Mutation γK194A—The results of a microarray-based proteolytic profiling assay suggested that plasmin has a strong preference for cleaving after Arg or Lys when the preceding residue (at the P2 position) is an aromatic residue Phe or Tyr, in addition to His (*i.e.* FR, HR, YR, FK, HK, YK) (17). These results are consistent with cleavage of the known and postulated substrates of plasmin (17). The assay also provided data for preferred residues at the P3 position. Several of these preferred tripeptide sequences are present in the γ subunit: γPYK^{106}, γIHK^{194}, γWYK^{237}, γSFK^{370}, and γSFK^{476}. On the basis of the plasmin-dependent 70-kDa form of γ, we predicted that plasmin cleaved at γIHK^{194}, just distal to the prostasin cleavage site at RKRK^{186}. To address this possibility, we examined whether plasmin could activate channels with a γK194A mutant. Whereas plasmin treatment of oocytes expressing wild-type ENaC significantly activated amiloride-sensitive currents, ENaCs containing a γK194A mutant were not activated by plasmin (Fig. 6). Consistent with this observation, we observed that plasmin treatment of oocytes expressing wild-type ENaC, but not ENaC containing the γK194A mutant, produced the plasmin-dependent 70-kDa fragment of γ when oocytes were exposed to plasmin (10 μg/ml) for 4 min (Fig. 3*A*, *lanes 2* and *3* (wild-type γ) and *lanes 4* and *5* (γK194A)). The non-cleaved 93-kDa and furin-cleaved 75-kDa forms of γ were present when ENaC with either wild-type γ or mutant γK194A was expressed in oocytes.

We next examined whether plasminogen is present in the urine of rats with proteinuria. Urine was collected from obese,

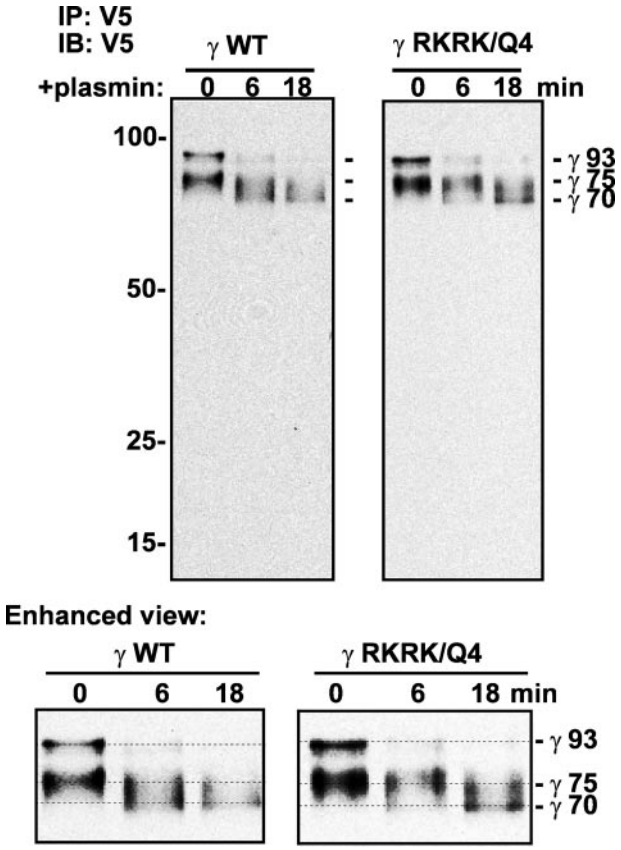

FIGURE 5. **Plasmin cleaves the γ subunit at a site distinct from the prostasin cleavage site.** MDCK cells were transiently transfected with α-Myc, β-FLAG, and either wild-type HA-γ-V5 (*γ WT*) or the HA-γRKRK^{186}/QQQQ-V5 mutant. ENaC was immunoprecipitated (*IP*) with anti-V5 antibodies from cell extracts, incubated with 15 μl of 20 μg/ml plasmin for 0, 6, or 18 min, and analyzed by immunoblotting (*IB*) with anti-V5 antibodies. Mobilities of the non-cleaved 93-kDa γ (*γ93*), furin-cleaved 75-kDa γ (*γ75*), and the new plasmin-dependent 70-kDa γ (*γ70*) fragments are indicated to the *right* of the gels. An enhanced version of the banding pattern is shown in the *lower panel* and includes three parallel lines that denote γ93, γ75, and γ70. *Numbers* to the left of the gels represent the mobility of Bio-Rad Precision Plus protein standards in kDa. The blot is representative of two independent experiments.

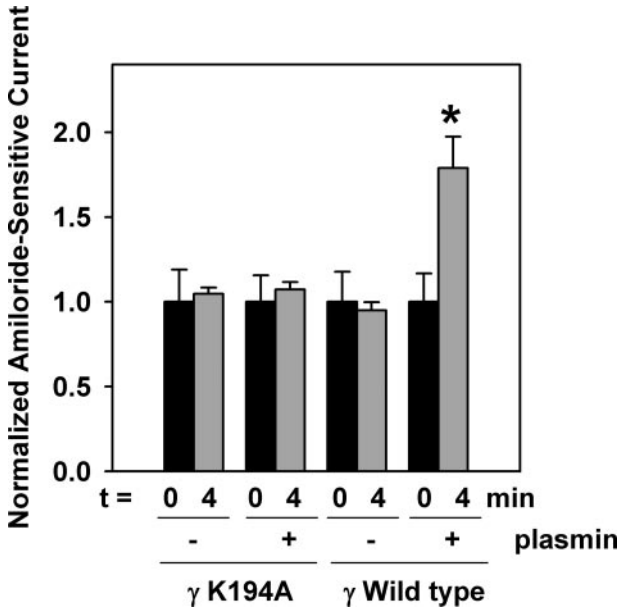

FIGURE 6. **ENaC containing γK194A is not activated by plasmin exposure.** Oocytes injected with cRNAs for non-tagged α and β and either the HA-γK194A-V5 mutant or wild-type HA-γ-V5 were analyzed by TEV before and after exposure to plasmin (10 μg/ml) for 4 min. Oocytes expressing the αβγK194A mutant or wild-type αβγ had stable amiloride-sensitive currents over 4 min of TEV recording in both the absence and presence of 10 μg/ml plasmin. For control oocytes expressing the αβγK194A mutant, amiloride-sensitive currents at base line were −2130 ± 400 nA (mean and S.E., $n = 19$). For oocytes exposed to plasmin, amiloride-sensitive currents at base line were −3380 ± 530 nA (mean and S.E., $n = 23$). Extracellular plasmin (10 μg/ml) for 4 min did not increase αβγK194A ENaC currents. Treatment of oocytes expressing wild-type αβγENaC for 4 min with plasmin (10 μg/ml) was used as a positive control. Amiloride-sensitive currents for control oocytes (−plasmin) at baseline were −2020 ± 360 nA (mean and S.E., $n = 18$). For oocytes exposed to plasmin, amiloride-sensitive currents at base line were −2020 ± 340 nA (mean and S.E., $n = 17$). Extracellular plasmin (10 μg/ml) for 4 min did increase wild-type αβγENaC currents by 1.8-fold. *, $p < 0.005$, plasmin treated *versus* control normalized currents at 4 min.

diabetic, and hypertensive ZSF1 rats and lean, nondiabetic, and hypertensive littermate controls. Obese ZSF1 rat urine contained 400 ± 52 mg of protein/kg/day ($n = 5$) compared with 53 ± 11 mg/kg/day ($n = 5$) for lean littermate controls ($p < 0.0005$). Equal volumes of urine were concentrated and subjected to SDS-PAGE and immunoblotting with an anti-plasminogen antibody. Although plasminogen and plasmin L-chain were readily detected in the urine of obese ZSF1 rats, they were largely absent in urine from lean littermate controls (Fig. 7).

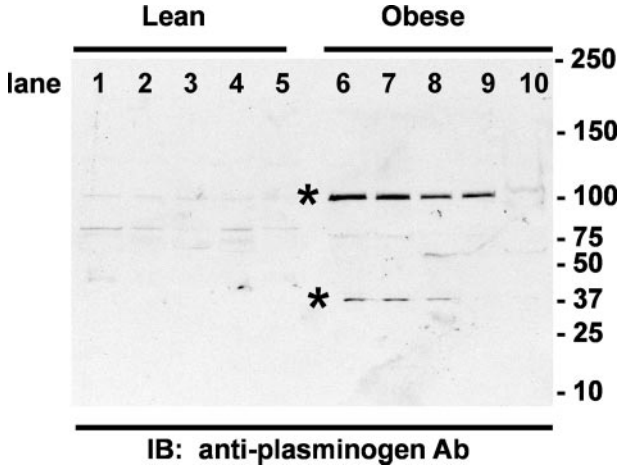

FIGURE 7. **Increased levels of plasminogen and plasmin are present in the urine of rats with metabolic syndrome.** Urine aliquots (0.15 ml) from five different control lean (*lanes 1–5*) and obese (*lanes 6–10*) rats were separately concentrated 10-fold and subjected to SDS-PAGE under reducing conditions for analysis by immunoblotting (*IB*) with anti-human plasminogen antibodies (*Ab*) as described under "Experimental Procedures." Note the plasminogen (~100 kDa) and plasmin L-chain (~37 kDa) in the urine of the obese rats (*asterisks*). *Numbers* to the *right* of the gel indicate the mobility of Bio-Rad Precision Plus protein standards in kDa on a 4–15% gel.

DISCUSSION

Our data show that extracellular plasmin activates ENaC currents in oocytes. Channel activation by plasmin is dependent on cleavage of the γ subunit at or near Lys^{194}. These observations are consistent with our proposed paradigm for the activation of ENaC by proteases, where activation occurs in a stepwise manner from (i) non-cleaved near silent channels to (ii) furin-processed channels that have an intermediate P_o, and potentially to (iii) channels that have been cleaved by furin and a second protease and have a high P_o (3, 5). We propose that extracellular plasmin activates furin-processed channels by

proteolytic processing of the γ subunit at γLys^{194} to release an inhibitory tract. Prostasin, elastase, kallikrein, and perhaps other proteases activate ENaC in a similar manner, by cleaving the γ subunit at a site distal to the defined furin cleavage site at γArg^{143}, releasing an inhibitory tract (Fig. 3*B*) (3, 5, 7–9).

In addition to activating ENaC, purified plasmin induces cleavage of ENaC expressed at the surface of both oocytes and MDCK cells. As purified plasmin also induces cleavage of immunopurified ENaC (Fig. 5), it is likely that plasmin directly cleaves the γ subunit and activates the channel. In addition to cleaving the γ subunit, it is possible that plasmin cleaves and activates other proteases that could process the γ subunit. Whereas plasmin may cleave the channel at other sites in addition to γLys^{194}, the lack of ENaC activation with the γK194A mutant suggests that plasmin-dependent cleavage at the site is required for channel activation in oocytes. Whether plasmin cleaves the channel at other sites and whether cleavage at these additional sites would affect channel activity in other cell types are questions that we have not addressed. For example, γPYK^{106} is a potential plasmin cleavage site. If plasmin cleaves the γ subunit at both Lys^{106} and Lys^{194}, it would potentially release the γ inhibitory tract we previously identified (5), providing a means of activating channels that have not been processed by furin.

Plasmin-dependent cleavage and activation of ENaC may be particularly relevant in human disorders associated with proteinuria and extracellular volume expansion. The pathogenesis of renal sodium retention in nephrotic syndrome is unclear. Recent studies suggest that enhanced ENaC activity has an important role in the renal Na^+ retention that has been observed in nephrotic syndrome (29–30). Plasminogen and its cleavage products are present in urine from a proteinuric rat model and not detected in urine from lean littermate controls. This observation is consistent with previous reports of differences in urinary plasminogen concentration found between humans with nephrotic syndrome and controls (18, 19). In the setting of nephrotic syndrome, plasminogen filtered by the glomerulus could be processed by urokinase released from cells that line the proximal and distal nephron to generate active plasmin in the region where ENaC resides (18–22), providing a mechanism for ENaC activation and renal Na^+ retention.

Note Added in Proof—While this manuscript was in press, we learned that Svenningsen and colleagues also have a manuscript in press in *J. Am. Soc. Nephrol.* demonstrating that plasmin cleaves and activates ENaC (31).

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