Urinary Plasmin Inhibits TRPV5 in Nephrotic-Range Proteinuria

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ABSTRACT

Urinary proteins that leak through the abnormal glomerulus in nephrotic syndrome may affect tubular transport by interacting with membrane transporters on the luminal side of tubular epithelial cells. Patients with nephrotic syndrome can develop nephrocalcinosis, which animal models suggest may develop from impaired transcellular Ca2+ reabsorption via TRPV5 in the distal convoluted tubule (DCT). In nephrotic-range proteinuria, filtered plasminogen reaches the luminal side of DCT, where it is cleaved into active plasmin by urokinase. In this study, we found that plasmin purified from the urine of patients with nephrotic-range proteinuria inhibits Ca2+ uptake in TRPV5-expressing human embryonic kidney 293 cells through the activation of protease-activated receptor-1 (PAR-1). Preincubation with a plasmin inhibitor, a PAR-1 antagonist, or a protein kinase C (PKC) inhibitor abolished the effect of plasmin on TRPV5. In addition, ablation of the PKC phosphorylation site S144 rendered TRPV5 resistant to the action of plasmin. Patch-clamp experiments showed that a decreased TRPV5 pore size and a reduced open probability accompany the plasmin-mediated reduction in Ca2+ uptake. Furthermore, high-resolution nuclear magnetic resonance spectroscopy demonstrated specific interactions between calmodulin and residues 133–154 of the N-terminus of TRPV5 for both wild-type and phosphorylated (S144pS) peptides. In summary, PAR-1 activation by plasmin induces PKC-mediated phosphorylation of TRPV5, thereby altering calmodulin-TRPV5 binding, resulting in decreased channel activity. These results indicate that urinary plasmin could contribute to the downstream effects of proteinuria on the tubulointerstitium by negatively modulating TRPV5.


In the kidney, the fine regulation of Ca2+ balance occurs through the activity of the epithelial Ca2+ channel TRPV5.1 TRPV5 is mostly expressed in the distal convoluted tubule (DCT) and connecting tubule of the nephron, where it constitutes the apical entry mechanism for transcellular Ca2+ reabsorption. TRPV5 is a constitutively active ion channel that bears unique electrophysiologic characteristics, including calmodulin (CaM) and Ca2+-dependent inactivation and high selectivity for Ca2+.2,3 The activity of TRPV5 is tightly controlled at multiple levels by an array of different factors, including parathyroid hormone and the serine protease tissue kallikrein. Both parathyroid hormone and tissue kallikrein initiate the phosphorylation of TRPV5 through the cAMP/protein kinase A (PKA) and phospholipase C (PLC)/diacylglycerol (DAG)/protein kinase C (PKC) signaling cascades, respectively.4,5

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Proteinuria is a hallmark of nephrotic syndrome, in which large plasma proteins pass through the disrupted glomerular basement membrane (GBM). Pathologic leakage of glomerular proteins causes multiple tubulointerstitial abnormalities, such as interstitial inflammation and eventually fibrosis, but does not affect tubular structure. However, recent data showed that tubular transport processes could be affected by direct effect of urinary protein on membrane transporters at the luminal side of the DCT, such as epithelial sodium channel. Whether urinary plasmin also can affect tubular Ca\(^{2+}\) handling and, if so, by which mechanisms, has not been established so far. This study aimed to investigate the effects and mechanism of urinary plasmin on TRPV5-mediated Ca\(^{2+}\) reabsorption.

**RESULTS**

**Plasmin in Nephrotic Urine Inhibits TRPV5-Mediated Ca\(^{2+}\) Influx**

To investigate the effect of plasmin on TRPV5-mediated Ca\(^{2+}\) transport, human embryonic kidney (HEK) 293 cells were transfected with TRPV5 and treated with 10 nM plasmin for 1 hour before radiotracer \(^{45}\)Ca\(^{2+}\) influx measurements. Plasmin inhibited TRPV5-mediated Ca\(^{2+}\) influx to an extent similar to that seen with ruthenium red, thus indicating completely inhibited TRPV5 activity (Figure 1A). Plasmin inhibits Ca\(^{2+}\) influx with a 50% inhibitory concentration of approximately 3 nM (Figure 1B) after at least 30 minutes of incubation (Supplemental Figure 1). The specific plasmin inhibitor \(\alpha_2\)-antiplasmin reversed this block (Figure 1A). Purified plasmin from the urine of five nephrotic patients mimicked the inhibitory effect compared with commercial plasmin. This urinary activity could be reversed by heat inactivation and \(\alpha_2\)-antiplasmin (Figure 1C). The presence of plasmin in urine samples and their activities are depicted in Figure 1D.

**Plasmin Does Not Affect TRPV5 Membrane Abundance**

Because plasmin has been reported to cleave the membrane-bound epithelial sodium channel, we investigated whether plasmin could exert its effects via cleavage of TRPV5 using cell surface biotinylation experiments. Figure 2A shows no change in plasma membrane or total expression of TRPV5 (protein input) after plasmin treatment (immunoblot intensities are shown in Figure 2B). The positive control tissue kallikrein could enhance TRPV5 membrane abundance without affecting the channel total expression (Figure 2A).

Patients with nephrotic syndrome (NS) have elevated serum plasminogen levels. After leakage into the urine, plasminogen is converted into active plasmin by tubular urokinase-type plasminogen activator and has recently been reported to regulate renal ion transport in nephrotic syndrome by effects on the epithelial sodium channel. Whether urinary plasmin also can affect tubular Ca\(^{2+}\) handling and, if so, by which mechanisms, has not been established so far. This study aimed to investigate the effects and mechanism of urinary plasmin on TRPV5-mediated Ca\(^{2+}\) reabsorption.
TRPV5 Inhibition by Plasmin Is Mediated by Protease-Activated Receptor-1

Because plasmin failed to cleave TRPV5 at the plasma membrane, we hypothesized that the reduced TRPV5 activity is mediated by a cell surface membrane receptor. Plasmin has been previously shown to bind the protease-activated receptor-1 (PAR-1), prostatin, and megalin.13–16 Therefore, we examined mRNA expressions of F2R, PRSS8, and LRP2, which encode PAR-1, prostatin, and megalin, respectively. Our results showed that only PAR-1 is endogenously expressed in HEK293 cells (Figure 3A). PAR-1 protein expression is shown by immunoblot (Figure 3B). Co-localization of PAR-1 and TRPV5 in late DCT and connecting tubules of mouse kidney cortex is depicted by immunohistochemistry (Figure 3C). Fura-2–based Ca²⁺ imaging experiments showed that PAR-1 was functional because it stimulated intracellular Ca²⁺ release (Figure 3D). The PAR-1 inhibitor SCH79797 and PAR-1 antibody prevented TRPV5 inhibition by plasmin, indicating a specific action of plasmin through PAR-1 (Figure 3E).

Plasmin-Induced Inhibition of TRPV5 Depends on PKC Phosphorylation of Serine-144 Residue

PAR-1 is a member of the G protein–coupled receptor family, which normally exerts its effect through the PKA17 or PLC/PKC pathways.18 We investigated whether these protein kinases are involved in plasmin-activated PAR-1 signaling. TRPV5 possesses three potential phosphorylation sites for PKA: One is on the N-terminus (Nter) and the other two are on the C-terminus (Cter).4 In addition, there are six sites for PKC: three sites on both Nter and Cter.5 HEK293 cells were transfected with Nter-PKA, Cter-PKA, or six-PKC phosphorylation site-mutated TRPV5. All PKA- and PKC-TRPV5 mutants were still able to transport Ca²⁺ to the same extent as wild-type TRPV5. However, plasmin failed to inhibit Ca²⁺ uptake in the six-PKC-phosphorylation site mutant, suggesting that at least one of these sites is responsible for plasmin action (Figure 4A). This finding prompted us to find the PKC phosphorylation site responsible for the inactivation of TRPV5. In combination with the Ca²⁺ uptake assay, serine (S) residue located in the ankyrin repeat domain 3 of the Nter mutated to alanine (A), S144A,5 was found to be unresponsive to plasmin (Figure 4C). The involvement of the PLC/PKC signaling pathway was further confirmed by establishing the reversal effect of PLC and PKC antagonists (U73122 and chelerythrine, respectively) on the inhibitory action of plasmin (Figure 4B).

Plasmin Specifically Decreases TRPV5-Mediated Ca²⁺ Current by Reducing Its Pore Size and Open Probability

The functional effect of plasmin on TRPV5-mediated Ca²⁺ current measured by patch-clamp technique was studied in HEK293 cells expressing TRPV5, by incubating cells for 1 hour with 10 nM plasmin. Before Ca²⁺ currents were measured, noninactivating inward-rectifying Na⁺ currents were recorded in the presence of 50 μM EDTA. Neither the amplitude of the Na⁺ current nor current-to-voltage relationship was affected by plasmin (Figure 5, A and B). However, in the presence of 10 mM 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) intracellularly, a significant decrease in the Ca²⁺ peak current from 1.02 ± 0.07–0.73 ± 0.06 nA/pF was observed in plasmin-treated group without affecting TRPV5 inactivation kinetics (Figure 5, C–E). This effect was not observed in the S144A mutant. Similar results were
seen in the presence of 50 μM ethylene glycol tetraacetic acid (Supplemental Figure 2).

To further substantiate the inhibitory effect of plasmin on the Ca^{2+}-mediated current, we performed sieving experiments after plasmin treatment to estimate changes in TRPV5 effective pore diameter by measuring relative permeability to organic monovalent cations of increasing size. Divalent-free intra- and extracellular solutions were used to avoid Mg^{2+} and Ca^{2+} block of monovalent currents. Starting with Na^{+} as the sole intra- and extracellular cation, TRPV5 currents reversed close to 0 mV. Subsequently, all Na^{+} in the extracellular solution was replaced by mono-, di-, tri-, or tetra-methyl substituents. Then permeability ratios of the substituents relative to Na^{+} (P_{X}/P_{Na}) from the bi-ionic reversal potential were determined. Figure 5F shows the permeability ratios of the different cations versus their estimated diameters obtained for TRPV5 treated with plasmin. Data points were fitted using a modified excluded volume equation:

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(P_{X}/P_{Na})^{1/2} = dP - dX/dP - dNa,
\]

where dP, dX, and dNa are diameters of the pore and specific cation X^{+} and Na^{+}, respectively. Plasmin-treated cells showed a significant decrease in the estimated pore diameter (mean ± SEM, 6.72 ± 0.07 and 6.18 ± 0.04 Å for control and plasmin-treated cells, respectively; P<0.05) (Figure 5G).

In different cell types, PKC activation via PAR-2 affected open probability of TRPV1.19,20 The cell-attached single-channel recordings keep intracellular second messengers and kinases intact, thereby making them suitable for investigating TRPV5 function in the present study. The recordings were made from HEK293 cells expressing TRPV5-wild type (WT) (Figure 6A) or TRPV5-S144A (Figure 6B). The averaged calculated slope conductance was 68 ± 4 picosiemens (pS) for TRPV5-WT and 72 ± 4 pS for TRPV5-WT preincubated with 10 nM plasmin for 1 hour (Figure 6B). Similarly, the averaged calculated slope conductance was 65 ± 3 pS for TRPV5-S144A and 66 ± 2 pS for TRPV5-S144A preincubated
with 10 nM plasmin for 1 hour (Figure 6D). In Figure 6, C and F, the averaged open probability upon plasmin incubation was assessed by averaging 10-second intervals for 1 minute using a holding potential of −80 mV (for representative traces, see Supplemental Figure 3). Plasmin significantly decreases the open probability in TRPV5-WT-expressing cells, but does not influence open probability in cells expressing TRPV5-S144A.

Calmodulin Selectively Binds TRPV5, and This Binding Is Affected by Phosphorylation of S144 in the N-Terminus of TRPV5

The effect of plasmin on TRPV5 has been shown to be highly specific for Ca\(^{2+}\). We decided, therefore, to test whether the ubiquitous Ca\(^{2+}\)-sensor CaM was required for plasmin-mediated TRPV5 inhibition. The Calmodulin Target Database (2002 Ikura Lab, Ontario Cancer Institute; http://calcium.uhnres.utoronto.ca) indicated that the specific region surrounding the highly conserved S144 amino acid holds the highest probability for potential CaM binding (Figure 7A). 15N-1H-heteronuclear single quantum coherence (HSQC) spectra of 15N-labeled CaM were measured by high-resolution nuclear magnetic resonance (NMR) spectroscopy under various concentrations of the synthetic peptides corresponding to residues 133–154 of Nter. In the presence of Ca\(^{2+}\), both WT and S144pS mutant peptides specifically bound CaM. As seen from the overlaid 15N-1H-HSQC spectra of CaM in Figure 7, B and C, CaM peaks shift in a similar fashion upon addition of the peptides. This indicates that both peptides interact with CaM in a generally similar way, with some slight differences. Namely, in case of the WT peptide, the intensity of the peaks remains the same in the course of the titration, which implies fast exchange (on the NMR time scale). However, in case of the S144pS mutant, the intensity of the shifted signals significantly decreases (e.g., I27, A57, D64), indicating that the CaM-peptide complex is now in intermediate exchange regime. Thus, phosphorylation of S144 clearly affects CaM-TRPV5 binding and alters its dynamic properties.

DISCUSSION

This study shows that plasmin present in the urine of patients with nephrotic syndrome inhibits TRPV5-mediated Ca\(^{2+}\) transport. This inhibition is mediated by PAR-1 through PKC phosphorylation of the Ca\(^{2+}\) channel at the S144 residue. Our conclusion is based on the following findings: (1) Plasmin, both the commercially available form and that concentrated from the urine of patients with nephrotic syndrome, inhibits TRPV5-mediated Ca\(^{2+}\) transport without affecting TRPV5 surface membrane expression; inhibition of Ca\(^{2+}\) transport could be prevented by the specific plasmin inhibitor α\(_2\)-antiplasmin and heat inactivation.
Plasmin activity requires an intact PAR-1/PLC/PKC signaling pathway, which phosphorylates TRPV5 at S144. Plasmin decreases TRPV5-mediated Ca\(^{2+}\) currents mainly by reducing the open probability of TRPV5 without affecting monovalent currents. Residues 133–154 of TRPV5 selectively bind CaM, and this binding is affected by phosphorylation of S144. Plasmin is well known for its action in fibrinolytic processes. Our data suggest that plasmin may be involved in modulating Ca\(^{2+}\) reabsorption in the kidney under pathophysiologic conditions, namely glomerular protein leakage. Plasmin has recently been identified as a crucial factor for Ca\(^{2+}\) homoeostasis and normal bone maintenance. Plasminogen-deficient mice manifest decreased trabecular and cortical bone density because of enhanced osteoclast activity and retarded osteoblast activity. In the kidney, plasmin is known to play a major role in the degradation and turnover of the extracellular matrix and especially the glomerular mesangial matrix. In addition, plasmin has long been hypothesized to be involved in renal stone formation because plasmin and urokinase-type plasminogen activator activities are decreased in stone-forming patients. In that study, van Aswegen and colleagues reported that urate inhibited urokinase/plasmin activities at a pH of 3.9 (in the presence of acetate) but is minimal under alkaline condition (in the absence of acetate). Therefore, it can be inferred that plasmin is still stable in our patients’ urine, with its pH of 7.5–7.7. Our laboratory previously showed that extracellular alkalization resulted in a pool of TRPV5-containing vesicles recruited to the cell surface. Our findings suggest that, once present at the cell surface, TRPV5 activity is inhibited by plasmin in nephritic urine via its receptor PAR-1. In vivo studies on Ca\(^{2+}\) balance, however, would be required to fully substantiate this assumption. Of note, in line with our data, pharmacologic reduction of proteinuria leads to a corresponding reduction in calciuria in clinical study.

Figure 6. Plasmin inhibits single-channel activity of TRPV5-WT but not TRPV5-S144A. (A and D) Cell-attached single-channel recordings were made from HEK293 cells expressing TRPV5-WT or TRPV5-S144A. Channel activity was elicited by step potentials varying from −100 to 80 mV for TRPV5-WT (A) and TRPV5-S144A (D). Downward currents indicate channel opening state. (B and E) Amplitude histograms were constructed from regions of the single-channel recordings and were fitted by three Gaussian functions corresponding to closed, one open, or two open levels. (C and F) The averaged open probability upon plasmin incubation was assessed by averaging 10-second intervals for 1 minute using a holding potential of −80 mV. *P<0.05 versus nonplasmin condition (n=5–7).
PAR-1 has been reported to couple to various heterotrimeric G proteins and conveys its signal through several kinases, including Src family tyrosine kinases, JNK, Rho kinases, mitogen-activated protein K, PKA, and PKC.17,19 Protein kinases have been found to differentially regulate ion channel gating and trafficking. Upon stimulation, PAR-1 enhances PLC-β-dependent hydrolysis of PIP2, which results in DAG and inositol trisphosphate production. DAG further stimulates PKC-dependent phosphorylation of TRPV5. In this study, we show that S299 and S654 double mutant is still responsive to plasmin effect (Supplemental Figure 4A). On the other hand, the S144 mutant is also stimulated by tissue kallikrein. Therefore, plasmin and tissue kallikrein induce PKC phosphorylation of TRPV5 at independent sites. 12-O-tetradecanoylphorbol-13-acetate has been previously shown to downregulate a, b, and e. Accordingly, the present data showed that 24-hour 12-O-tetradecanoylphorbol-13-acetate treatment prevented the effects of both tissue kallikrein and plasmin (Supplemental Figure 4B). The specific subtype of PKC involved in this process has not yet been characterized. However, PKC-α and PKC-ε have been reported to mediate PAR-1 signaling,26 and we would like to suggest that the Ca2+-independent PKC-ε isoform is more likely to be involved in this pathway because plasmin-mediated PAR-1 activation is sufficient to inhibit TRPV5 in the presence of intracellular Ca2+ chelator BAPTA. The atypical PKC isoforms were probably not involved because the effect of plasmin is DAG dependent (Supplemental Figure 4B).

TRPV5 is the most Ca2+-selective member of the TRP superfamily involved in Ca2+ reabsorption in renal epithelia, as shown by knockout mice studies.27 TRPV5 activity depends on calciotropic hormones to ensure proper Ca2+ reabsorption,28 but other molecules are also involved in the TRPV5 regulation. Activation of PKC by tissue kallikrein via the bradykinin receptor phosphorylates TRPV5 at S299 and S654, resulting in an inhibition of channel internalization.23 This process retains

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**Figure 7.** S144 residue located on the third ankyrin repeat domain-3 (ANK3) of the N-ter-TRPV5 is conserved among mammalian species. The potential CaM-binding sequence (RGASVSRARA) is depicted (A). 15N-1H-HSQC spectra of 15N-cALM in various concentrations of the synthetic peptides: (B) WT, (C) S144P mutant. Calmodulin-to-peptide molar ratios are represented as follows: black, 1:0; red, 1:1; blue, 1:2. Each peak corresponds to a unique proton attached to a 15N nucleus. Spectra were acquired at 308 K (600 MHz Bruker Avance III spectrometer).
the channel at the plasma membrane, thus increasing Ca\textsuperscript{2+} reabsorption. A similar effect of plasmin on TRPV5 surface expression was, however, not observed.

Electrophysiologic analysis of HEK293 cells expressing TRPV5 showed that plasmin specifically decreased Ca\textsuperscript{2+} permeability but had no effect on Na\textsuperscript{+} permeability. This can be explained by examining the selectivity filter of Ca\textsuperscript{2+} channels, which have the terminal carboxyl side chains of their aspartates and glutamates in the permeation pathway, directly interacting with passing Ca\textsuperscript{2+} ions.\textsuperscript{29,30} This structural organization is thought to be flexible, and, therefore, its configuration can change as a consequence of rearrangements of the tertiary and quaternary structure of the channel. Here, we have shown that phosphorylation of the S144 amino acid in the N\textsubscript{ter} of TRPV5 is required to observe the inhibitory effect of plasmin. We can, therefore, infer an alteration of pore structure, probably through changes in ion-ion interactions in the pore. This notion is supported by the observation that the effective pore diameter of TRPV5 (6.18 Å) after plasmin treatment is smaller than the hydrated size of Ca\textsuperscript{2+} ion (6.2 Å).\textsuperscript{31} At present it is unclear whether this reduction contributed to the diminished TRPV5 activity. Further analysis suggests that our reduction in Ca\textsuperscript{2+} transport is probably a consequence of a decrease in open probability induced by plasmin. This observation further suggests a role of plasmin in regulating Ca\textsuperscript{2+} reabsorption in the context of nephrotic syndrome because the glomerular protein leakage allows this enzyme to enter into the pro-urine to reach the DCT.

Because of the specific effect of plasmin on TRPV5 Ca\textsuperscript{2+} current and open probability, we hypothesized that another player could be involved in the plasmin-activated pathway. CaM has been recently shown to enhance Ca\textsuperscript{2+}-dependent TRPV5 inactivation by binding to the distal region of the C\textsubscript{ter} domain.\textsuperscript{32} The phosphorylation of T709 by PTH (via the PKA pathway) impedes CaM binding to the C\textsubscript{ter} and subsequently increases the open probability of TRPV5.\textsuperscript{32} CaM can be activated by Ca\textsuperscript{2+} influx via TRPV5\textsuperscript{32} or the release of Ca\textsuperscript{2+} from intracellular stores.\textsuperscript{33} During the resting state, intracellular free Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]) is relatively low and CaM remains inactive. Once [Ca\textsuperscript{2+}]\textsubscript{i} rises, Ca\textsuperscript{2+} binds to CaM via Ca\textsuperscript{2+}-binding EF-hand structures,\textsuperscript{2} which in turn stretches CaM as each pair of EF hands exposes a hydrophobic patch available to bind target amino acid sequences.\textsuperscript{34} One of the consequences is the inhibition of Ca\textsuperscript{2+} channel activities.\textsuperscript{35} In addition to binding of CaM to the C\textsubscript{ter} domain, several studies have shown that CaM is able to bind the N\textsubscript{ter} domain of TRPV5 in a Ca\textsuperscript{2+}-dependent manner,\textsuperscript{32,36,37} although no functional experiments have been performed to assess the effects of N\textsubscript{ter} binding on channel activity. In fact, CaM has recently been reported to bind in vitro to the N\textsubscript{ter} (residues 133–154 and 310–330).\textsuperscript{38}

The present study revealed that plasmin-induced S144 phosphorylation modifies CaM binding affinity of the surrounding region of S144, in addition to a decrease of TRPV5 open probability and changing the channel pore diameter (Figure 8).

In conclusion, this study demonstrates that plasmin inhibits TRPV5 activity through the activation of the PAR-1 receptor via a PLC/PKC-dependent pathway. PKC activation results in phosphorylation of the S144 residue and consequently inhibits TRPV5 activity by decreasing its open probability and pore diameter. Urinary plasmin, by inhibiting TRPV5 activity, could potentially be involved in disturbances in renal Ca\textsuperscript{2+} handling found in nephrotic patients and may play a role in the tubulo-toxic effects of proteinuric urine.

**CONCISE METHODS**

**Collection of Urine Samples**

Twenty-four-hour urine samples were collected from five nephrotic patients (all male; mean age, 53 ± 2.6 years; mean creatinine clearance, 24 ± 9 ml/min) before their visits to the outpatient nephrology clinic. Their underlying diseases proven by biopsies were myeloperoxidase-ANCA–associated GN, IgA nephropathy, membranous nephropathy, FSGS, and diabetic nephropathy. All patients were overtly proteinuric (i.e., the median urinary protein excretion was 6.5 [interquartile range, 5.3–8.2] g/d). Collections were performed at University Medical Centre Groningen, and all patients provided informed consent.

**Plasmin Purification and Activity Assay**

Urine samples were dialyzed in Spectra/Por dialysis membrane (Spectrum Laboratories) for 12 hours with dialysis buffer (10 mM Tris HCl [pH, 8.1] and 5 mM EDTA). Urine precipitates were purified with high-affinity ion exchange chromatography with mono-Q column based on the theoretical isoelectric point of plasmin (7.08; http://expasy.org/tools/pi_tool.html). Urine samples were injected in the column with buffer A

![](image)

**Figure 8.** Molecular model of plasmin action on TRPV5 in nephrotic syndrome. The basal level of TRPV5-dependent Ca\textsuperscript{2+} influx is constitutively established by the endogenous CaM binding (A). Plasmin (PL) from nephrotic urine catalyzes PAR-1, promoting S144 phosphorylation by PKC. This results in decreased open probability (NPO) and pore size of TRPV5 and modification of CaM binding to the CaM-binding sequence (B).
(10 mM Tris [pH, 8.1] and 5 mM EDTA) and eluted with buffer B (10 mM Tris [pH, 8.1], 5 mM EDTA, and 1 M NaCl). Chromatography fractions were concentrated with 50K Amicon Ultra Centrifugal Filter Units (Millipore), and the presence of plasmin was determined by immunoblotting. Plasmin activity was measured with SensoLyte AFC plasmin activity assay kit (AnaSpec) according to a provided protocol.

**PCR Analysis**

To evaluate mRNA expression of F2R, PRSS8, and LRP2 (encoding PAR-1, prostein, and megalin, respectively), total RNA was extracted from HEK293 cells using TriZol Total RNA Isolation Reagent (Life Technologies BRL, the Netherlands) according to the manufacturer’s protocol. The obtained RNA was subjected to deoxyribonuclease treatment (Promega) to prevent genomic DNA contamination. Thereafter, 2 μg of RNA was reverse transcribed by Moloney-murine leukemia virus-reverse transcription (Invitrogen, the Netherlands). The cDNA was used to determine mRNA expression levels by PCR of the target genes of interest and of the housekeeping gene GAPDH as an endogenous control. Primers targeting the genes of interest were designed using the computer program Primer3 (version 0.4.0) and are listed in Table 1.

**DNA Constructs and Cell Culture**

TRPV5 pCINeo/internal ribosome entry site-green fluorescent protein (IRES-GFP) constructs were generated, as described previously. Single and combined PKC mutants were generated by alanine substitution of the six putative phosphorylation sites of TRPV5 (S144A, S299A, S316A, S654A, S664A, S698A) using the six putative phosphorylation sites of TRPV5 (S144A, S299A, S316A, S654A, S664A, S698A) using

Directed Mutagenesis kit, Stratagene). HEK293 cells were transfected with TRPV5-HA pCINeo/IRES-GFP constructs. Ca2+ uptake was determined in uptake medium (110 mM NaCl, 5 mM KCl, 1.2 mM MgCl2, 0.1 mM CaCl2, 10 mM Na-acetate, 2 mM NaH2PO4, and 20 mM HEPES–Tris [pH, 7.4]), supplemented with 10 μM felodipine, 10 μM methoxy-verapamil, and 1 μM CaCl2 for 10 minutes at 37°C. Each well was washed extensively with stop buffer (110 mM NaCl, 5 mM KCl, 1.2 mM MgCl2, 0.5 mM CaCl2, 1.5 mM LaCl3, 10 mM Na-acetate, 20 mM HEPES–Tris [pH, 7.4]) at 4°C, incubated with 0.05% wt/vol SDS, and the lysates were counted for radioactivity using liquid scintillation.

**45Ca2+ Uptake Assay**

HEK293 cells were transfected with TRPV5-HA pCINeo/IRES-GFP constructs. Ca2+ uptake was determined in uptake medium (110 mM NaCl, 5 mM KCl, 1.2 mM MgCl2, 0.1 mM CaCl2, 10 mM Na-acetate, 2 mM NaH2PO4, and 20 mM HEPES–Tris [pH, 7.4]), supplemented with 10 μM felodipine, 10 μM methoxy-verapamil, and 1 μM CaCl2 for 10 minutes at 37°C. Each well was washed extensively with stop buffer (110 mM NaCl, 5 mM KCl, 1.2 mM MgCl2, 0.5 mM CaCl2, 1.5 mM LaCl3, 10 mM Na-acetate, 20 mM HEPES–Tris [pH, 7.4]) at 4°C, incubated with 0.05% wt/vol SDS, and the lysates were counted for radioactivity using liquid scintillation.

**Cell Surface Biotinylation and Immunoblotting**

HEK293 cells (9×10⁴ cells/cm²) were plated and transfected with 15 μg TRPV5 pCINeo/IRES-GFP or pCINeo/IRES-GFP in poly-l-lysine (Sigma) coated 10-cm dishes. At 48 hours after transfection, cells were incubated for 1 hour with 10 nM plasmin and 50 nM α2-antiplasmin inhibitor. Cells were homogenized in 1 ml lysis buffer, as described previously using the NHS-LC-LC-biotin (Pierce, the Netherlands). Finally, biotinylated proteins were precipitated using NeutrAvidin beads (Pierce). TRPV5 expression was analyzed by immunoblotting for the precipitates (plasma membrane fraction) and for the total cell lysates using the guinea-pig antirabbit TRPV5 antibody.

**Ca2+ Imaging Using Fura-2/Acetoxymethyl**

HEK293 cells were seeded on fibronectin-coated coverslips. After 24 hours, cells were loaded with 3 μM fura-2-acetoxymethyl and 0.01% (vol/vol) Pluronic F-129 in DMEM medium at 37°C for 20 minutes. After loading, cells were washed twice with PBS and allowed to equilibrate at 37°C for another 10 minutes in 132 mM NaCl, 4.2 mM KCl, 1.0 mM MgCl2, 5.5 mM d-glucose, 10 mM EDTA, and 10 mM HEPES, and titrated to a pH of 7.4 with Tris. Plasmin with final a concentration of 10 nM was added directly to buffer solution. Changes in intracellular Ca2+ concentration ([Ca2+]) were monitored with fura-2 excited at 340 and 380 nm using a monochromator (Polychrome IV, Germany). All measurements were performed at room temperature. Details of this experiment were described previously.

**Immunohistochemistry**

Mouse kidney sections were incubated for 16 hours at 4°C with rabbit polyclonal antibody against PAR-1 (1:100). To visualize PAR-1, goat antirabbit Alexa Fluor 488-conjugated antibody (1:300) (Molecular Probes) was used. TRPV5 staining has been performed as previously described. All negative controls, including sections incubated with preimmune serum or conjugated antibodies solely, were devoid of any staining.

**Electrophysiology and Solutions**

Patch-clamp experiments were performed under the whole-cell configuration using an EPC-9 patch-clamp amplifier controlled by the Pulse software (HEKA Elektronik, Germany). Borosilicate patch pipettes had a resistance between 3 and 4 MΩ after being filled with the intracellular solutions. Series resistance (3–10 MΩ) was continuously monitored with the automatic capacitance compensation of Pulse software. The extracellular solution consisted of (in mM) 150 NaCl, 6 CsCl, 10 glucose, 10 HEPES, 44 mannitol, and 0.05 EDTA (divalent-free solution with EDTA [pH, 7.4] and NaOH). The intracellular solution included (in mM) 150 CsCl, 100 aspartate, 1 MgCl2, 10 ATP, 1 Na2ATP, and 10 HEPES.

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<tr>
<td>GAPDH</td>
<td>GCTGGACCCGAGCAGATTACC</td>
<td>CTTCTGGTTGCCAGTGGTC</td>
<td>650</td>
</tr>
</tbody>
</table>

F2R, coagulation factor II receptor gene; PRSS8, serine protease 8 gene; LRP2, low-density lipoprotein receptor-related protein 2 gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase gene.
(pH, 7.2 CsOH). For the recording of Ca\(^{2+}\) current, 10 mM CaCl\(_2\) was added to the extracellular solution. Cells were exposed for a maximum of 1 minute to a Krebs solution containing 1 mM Ca\(^{2+}\) before sealing the patch pipette to the cell. The analysis and display of patch-clamp data were performed using Igor Pro software (WaveMetrics).

**NMR Spectroscopy**

Titration of \(^{15}\)N-labeled CaM by nonlabeled peptides was done as previously described.\(^ {32,38}\) Briefly, \(^{15}\)N-labeled CaM was titrated by synthetic peptides with CaM-to-peptide molar ratios ranging from 1:0–1:3 in steps of 0.5. Before titrations, both CaM and peptides were dialyzed against the same buffer (1/100 of 50 mM KCl, 10 mM CaCl\(_2\), 20 mM Tris [pH, 7.0], HCl), freeze-dried and reconstituted in the same buffer. D\(_2\)O and NaN\(_3\) (both from Sigma) were added to the NMR samples to the final concentrations of 5%–7% (vol/vol) and 0.01% (vol/vol), respectively. The concentration of CaM in the samples was 0.4 mM. Spectra were acquired at 308 K, 600 MHz Bruker Avance III spectrometer.

**Compounds**

Plasmin, plasminogen, and \(\alpha_2\)-antiplasmin inhibitor were purchased from Sigma. PAR-1 antagonist (SCH79797) was from Axon Medchem (the Netherlands). A polyclonal PAR-1 antibody (rabbit IgG) was from Santa Cruz Biotechnology. A polyclonal plasminogen antibody (goat IgG) was obtained from Abcam (United Kingdom). Chelerythrine was from Research Biochemicals International (Germany). \(^{15}\)NH\(_4\)Cl was from Buchem BV (the Netherlands). Synthetic peptides corresponding to the residues 133–154 of hTRPV5 (WT and with phosphorylated S144, S144pS) were purchased from GenicBio Limited (China).

**Statistical Analyses**

If not specified otherwise, the data are expressed as mean ± SEM. Multiple sets of data were compared by ANOVA. The significant differences between the means of two groups were analyzed by unpaired t test, and multiple comparisons between groups were performed by Tukey post hoc analysis. The level of statistical significance is P<0.05. All data were analyzed by GraphPad Prism (version 4.0c for Mac OS X; GraphPad Software).

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**DISCLOSURES**

None.

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