Renal Proteinase-activated Receptor 2, a New Actor in the Control of Blood Pressure and Plasma Potassium Level*

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Background: The function of proteinase-activated receptor 2 (PAR2) in the distal nephron remains unknown.

Results: PAR2 activation increases electroneutral sodium reabsorption and inhibits potassium secretion in collecting ducts and thereby controls blood pressure and plasma potassium.

Conclusion: PAR2 controls sodium and potassium homeostasis.

Significance: PAR2 is a new actor of aldosterone paradox but also an aldosterone-independent modulator of blood pressure and plasma potassium.

Proteinase-activated receptor 2 (PAR2) is a G protein-coupled membrane receptor that is activated upon cleavage of its extracellular N-terminal domain by trypsin and related proteases. PAR2 is expressed in kidney collecting ducts, a main site of control of Na⁺ and K⁺ homeostasis, but its function remains unknown. We evaluated whether and how PAR2 might control electrolyte transport in collecting ducts, and thereby participate in the regulation of blood pressure and plasma K⁺ concentration. PAR2 is expressed at the basolateral border of principal and intercalated cells of the collecting duct where it inhibits K⁺ secretion and stimulates Na⁺ reabsorption, respectively. Invalidation of PAR2 gene impairs the ability of the kidney to control Na⁺ and K⁺ balance and promotes hypotension and hypokalemia in response to Na⁺ and K⁺ depletion, respectively. This study not only reveals a new role of proteases in the control of blood pressure and plasma potassium level, but it also identifies a second membrane receptor, after angiotensin 2 receptor, that differentially controls sodium reabsorption and potassium secretion in the late distal tubule. Conversely to angiotensin 2 receptor, PAR2 is involved in the regulation of sodium and potassium balance in the context of either stimulation or non-stimulation of the renin/angiotensin/aldosterone system. Therefore PAR2 appears not only as a new actor of the aldosterone paradox, but also as an aldosterone-independent modulator of blood pressure and plasma potassium.

Hypertension affects 15% of the population worldwide and is annually responsible for 5 million deaths from stroke, myocardial infarction, and heart and kidney failure. The pathophysiology of hypertension remains obscure. Except for few Mendelian forms of the disease, it appears as a complex trait related to a multitude of targets. Extracellular fluid volume and arterial pressure are mainly determined by the capacity of the kidneys to match the excretion of Na⁺ to its dietary intake (1, 2). Within the kidney, the aldosterone-sensitive distal nephron (ASDN), which includes the distal convoluted tubule (DCT), the connecting duct (CNT), and the cortical collecting duct (CCD), is a main site of control of Na⁺ excretion. Accordingly, most alterations of Na⁺ reabsorption along the ASDN are associated with hypo- or hypertension.

Proteases play important roles in the regulation of Na⁺ transport in the ASDN and blood pressure. Firstly, proteases are necessary for the maturation of hormones such as angiotensin 2 (e.g. renin and angiotensin-converting enzyme), bradykinin (e.g. kallikrein), and atrial natriuretic peptide (e.g. corin), which exert a strong control on Na⁺ excretion and on blood pressure. Secondly, serine proteases such as prostanin and kallikrein exert a paracrine control of sodium reabsorption by moderate proteolysis of the epithelial Na⁺ channel ENaC, a main actor of Na⁺ reabsorption in the late ASDN. Proteases are also responsible for the activation of the G protein-coupled membrane receptor PAR2 (for proteinase-activated receptor 2) (3) through cleavage of its N-terminal domain, which unmasks a tethered ligand. PAR2 is widely expressed in the body where it participates in inflammatory processes (4), but it is also known to modulate ion transport in several epithelia (5). PAR2 is expressed along the nephron, including the ASDN, and we recently reported that it controls Na⁺ transport in the nephron segment located upstream of the ASDN (6). Our first aim was to

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2 The abbreviations used are: ASDN, aldosterone-sensitive distal nephron; DCT, distal convoluted tubule; CNT, connecting duct; CCD, cortical collecting duct; PAR2, proteinase-activated receptor 2; PC, principal cell; IC, intercalated cell; ROMK, renal outer medullary potassium channel; ENaC, epithelial Na⁺ channel; TRITC, tetramethylrhodamine isothiocyanate; AP, agonist peptide; F-AP, furoylated AP derivative; HCTZ, hydrochlorothiazide; AVP, vasopressin; EGFP, enhanced green fluorescent protein; All, angiotensin 2; PDt, transepithelial voltage.
evaluate the role of PAR2 in the control of Na\(^+\) transport in the CCD, the only part of ASDN accessible to ex vivo investigation of Na\(^+\) transport, and the impact of this regulation on Na\(^+\) homeostasis and blood pressure.

CCDs and CNTs are made of principal and intercalated cells (PCs and ICs), which both participate in Na\(^+\) reabsorption. In PCs, electrogenic Na\(^+\) reabsorption is mediated by basolateral Na-K-ATPase and apical amiloride-sensitive ENaC. ENaC-mediated Na\(^+\) entry depolarizes the apical membrane, which provides a driving force for secretion of K\(^+\), mainly via ROMK channels. In ICs, apical entry of NaCl is mediated via the coupling between the Cl\(^-\)/HCO\(_3\)^- exchanger pendrin (Slc26a4) and a Na\(^+\)-dependent Na\(^+\),HCO\(_3\)^-/Cl\(^-\) exchanger (Slc4a8) (7). Cl\(^-\) exits ICs at the basolateral membrane via Cl\(^-\) channels, but the mechanism of basolateral Na\(^+\) exit and the motor that energizes the whole process have not yet been elucidated. This system is electroneutral and is sensitive to thiazide. Because these two Na\(^+\) reabsorption systems participate in the control of distinct functions, i.e. control of both K\(^+\) balance and volemia through Na\(^+\)/K\(^+\) exchange by PCs versus exclusive control of volemia through NaCl reabsorption by ICs, we looked at the expression of PAR2 in PCs and ICs and at its effects on their respective pathways of cation transport.

Results show that PAR2 is expressed at the basolateral membrane of ICs and PCs where the main effects of its activation are to stimulate electroneutral NaCl reabsorption and to inhibit K\(^+\) secretion, respectively. Through these actions, PAR2 participates in the control of blood pressure and plasma K\(^+\) concentration.

**EXPERIMENTAL PROCEDURES**

**Animals**—Experiments were performed on male Sprague-Dawley rats (Charles River Laboratories, L’Arbresle, France), male PAR2 gene-targeted mice (PAR2\(^{-/-}\), The Jackson Laboratory, Bar Harbor, ME), mice expressing EGFP in intercalated cells (8), and wild type control littermates. Animals were fed ad libitum either the standard rodent chow containing 0.3% Na\(^+\) and 0.6% K\(^+\) or a Na\(^+\)-deficient (0.01% Na\(^+\)) or a K\(^+\)-deficient diet (<0.001% K\(^+\)) with free access to tap water. Protocols were approved by the local ethic committee at the Centre de Recherches des Cordeliers, and experiments were performed by an authorized person.

**Metabolic Studies**—After 4 days of acclimatization to individual metabolic cages (Tecniplast, Limonest, France), urine was collected and Na\(^+\), K\(^+\), Ca\(^{2+}\) and creatinine concentrations were determined (Konelab 20i, Thermo, Cergy, France). For blood pressure measurements in conscious, restrained mice by the tail-cuff method (BP-2000, Visitech system, Biosel, Vitrolles, France), animals were adapted for 3 days before the onset of the experiment. Plasma K\(^+\) was measured (ABL77, Radiometer, Bronshoj, Denmark) on blood withdrawn from the retro-orbital sinus under isoflurane anesthesia.

**CCD Microdissection**—CCDs were dissected either from fresh kidney slices (microperfusion and [Ca\(^{2+}\)]\(_i\), measurements) or after a mild treatment with liberase (Blendozyme 2, Roche Diagnostics, Meylan, France) (Na,K-ATPase measurement, immunoblotting, immunohistochemistry, and RT-PCR) (6).

**In Vitro Microperfusion**—Unless indicated otherwise, CCDs were perfused at 37 °C under symmetrical conditions (7), with bath and perfusate containing (in mm): 118 NaCl, 23 NaHCO\(_3\), 1.2 MgSO\(_4\), 2 K\(_2\)HPO\(_4\), 2 calcium lactate, 1 sodium citrate, 5.5 glucose, 5 alanine, pH 7.4 (bath continuously gassed with 95% O\(_2\)/5% CO\(_2\)). Transepithelial voltage (PD\(_{te}\)) was measured via microelectrodes connected through an Ag/AgCl half-cell to an electrometer in tubules perfused at a high rate (~15 nl/min). Conversely, for flux measurement, we used a low perfusion rate (~2 nl/min), and creatinine 12 mM was added to both bath and perfusion medium to determine water flux. Concentrations of Na\(^+\), K\(^+\), and creatinine were determined by HPLC, and ion flux (J\(_{X}^+\)) was calculated as

\[
J_X^+ = \frac{\left[(X^+)_{p} \times V_p\right] - \left[(X^+)_{c} \times V_c\right]}{L \times t} \quad \text{(Eq. 1)}
\]

where \([X^+]_{p}\) and \([X^+]_{c}\) are the concentration of \(X^+\) in the perfusate and collection, respectively, \(V_p\) and \(V_c\) are the perfusion and collection rates, respectively, \(L\) is the tubule length, and \(t\) is the collection time. For each tubule, Na\(^+\) and K\(^+\) flux were calculated as the mean of four successive 10–15-min collection periods.

**Measurement of [Ca\(^{2+}\)]\(_i\)**—Intracellular calcium concentration was determined on single CCDs either at the level of a mixed population of PCs and ICs or at the single cell level. For mixed cell setup, [Ca\(^{2+}\)]\(_i\), was determined by the fura 2 fluorescence, as described previously (9). CCDs loaded with acetoxy-methyl ester of fura 2 (10 μM, 1 h at room temperature) were transferred to a perfusion chamber and immobilized. The peritubular fluid maintained at 37 °C was continuously exchanged at a rate of ~10 ml/min. After a 5–10 min equilibration, fura 2 fluorescence of ~15 cells was measured with a standard photometric setup (MSP 21, Zeiss). Tubule autofluorescence was subtracted from the fluorescence intensities of fura 2 at 340 and 380 nm. [Ca\(^{2+}\)]\(_i\) values were calculated as described previously (9).

For single cell measurements, CCDs were perfused in vitro, and intercalated cells were labeled by adding fluorescein-labeled peanut lectin (PNA, Vector Laboratories) to the luminal perfusate for 5 min and detected with excitation and emission wavelengths of 440 and 530 nm, respectively. Cells were loaded with Fluo-4 acetoxyethyl ester (20 μM, 20 min at room temperature) added to the bath solution. The loading solution was then washed out by initiation of bath flow, and the tubule was equilibrated with dye-free bath solution for 10 min. The bath flow was 20 ml/min, and the perfusion rate was ~20 nl/min. Fluo-4 was excited every 2 s at 488 nm with a light-emitting diode (OptoLED, Cairn Research, Faversham, UK). Emitted light at 510-nm filter was focused onto an EM-CCD camera (iXon, Andor Technology, Belfast, Ireland) and digitized for further analysis. For each tubule, 3–4 ICs and 3–4 PCs were analyzed, and the mean gray was measured with the Andor IQ software (Andor Technology). Background fluorescence was subtracted from fluorescence intensity to obtain the intensity of intracellular fluorescence. The Fluo-4-measured [Ca\(^{2+}\)]\(_i\) changes were expressed relative to stable base-line values.

**Na,K-ATPase Assay**—Na,K-ATPase activity of isolated CCDs was determined as described previously (10).
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RNA Extraction and RT-PCR—RNAs were extracted from 40–60 mouse CCDs using an RNeasy micro kit (Qiagen, Hilden, Germany) and reverse transcribed using first strand cDNA synthesis kit for RT-PCR (Roche Diagnostics), according to the manufacturers’ protocols. Real time PCR was performed with a LightCycler 480 SYBR Green I master quantitative PCR kit (Roche Diagnostics) according to the manufacturer’s protocol. Specific mouse PAR2 primers were designed using Probe-Design (Roche Diagnostics): sense, 3′-gggcatcgactcat-5′; antisense, 3′-ccgacacttccgacgca-5′.

Immunoblotting—Pools of 50–80 CCDs were solubilized in Laemmli buffer, and proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (GE Healthcare). After blocking, blots were successively incubated with anti-ERK antibody (p44/42 MAP kinase antibody, Cell Signaling Technology, 1/500) and anti-rabbit IgG antibody coupled to horseradish peroxidase (Promega France, Charbonnières, France) and revealed with the Western Lightning chemiluminescence reagent Plus (PerkinElmer Life Sciences). After stripping, membranes were incubated with anti-phospho-ERK antibody (phospho-44/42 MAP kinase antibody, Cell Signaling Technology, 1/500) and anti-rabbit IgG antibody coupled to horseradish peroxidase (Promega France, Charbonnières, France) and revealed with the Western Lightning chemiluminescence reagent Plus (PerkinElmer Life Sciences).

Immunohistochemistry—CCDs were transferred to Super-frost Gold+ glass slides, fixed with 4% paraformaldehyde, incubated for 20 min at room temperature in 100 mm glycerin, and permeabilized for 30 s with 0.1% Triton. After blocking, slides were incubated with anti-PAR2 antibody (APR-032, Alomone Labs, 1/200, 1 h at room temperature), rinsed and incubated with TRITC- or FITC-coupled anti-rabbit IgG (1/500, 1 h at room temperature), and observed on a confocal microscope (×40, Zeiss observer.Z1, LSM710).

Statistics—Results are expressed as means ± S.E. from several animals. Comparison between groups was performed either by unpaired t test or by variance analysis followed by Fisher’s protected least significant difference test, as appropriate.

RESULTS

PAR2 Expression in CCD—The specificity of the anti-PAR2 antibody used in this study was demonstrated by showing immunolabeling in CCDs from wild type (WT) but not PAR2−/− mice (Fig. 1A). In rat CCDs, immunolabeling was seen at the basolateral but not the apical pole of cells (Fig. 1B). Labeling of CCDs from mice expressing EGFP specifically in ICs revealed that both EGFP-negative and EGFP-positive cells, i.e. PCs and ICs, express PAR2 (Fig. 1C).

Activity of PAR2 was attested by showing that basolateral but not luminal addition of trypsin to rat CCDs dose-dependently increased [Ca2+]i (Fig. 1, D–G). Trypsin also increased [Ca2+]i in CCDs from WT but not PAR2−/− mice (not shown). The effect of trypsin on [Ca2+]i was mimicked by the PAR2-specific agonist peptide SLIGRL-NH2 (AP), but not by the inactive reverse peptide LGIRL-NH2 (Fig. 1, F–G). The furoylated AP derivative (2-furoyl-LIGRL-NH2, F-AP) (11) mimicked trypsin and AP effects. The PAR2-induced increase in [Ca2+]i, originated from both PCs and ICs (Fig. 1, H and I).

PAR2 and Sodium Reabsorption—As reported previously (12), microperfused rat CCDs showed no net transport of Na+ at base line, but basolateral addition of trypsin induced an Na+ reabsorption flux (Fig. 2A). It also induced Na+ reabsorption in CCDs from WT but not PAR2−/− mice (Fig. 2B). Trypsin-induced Na+ flux in rat CCDs was markedly inhibited by hydrochlorothiazide (HCTZ), whereas amiloride had a moderate and nonsignificant effect (Fig. 2A).

To confirm or invalidate the effect of PAR2 on amiloride-sensitive Na+ transport, we looked for changes in transepithelial voltage (PDve), which is a more sensitive readout of electrogenic Na+ reabsorption than amiloride-sensitive Na+ flux. Trypsin induced a transient depolarization of rat CCDs followed by a small but sustained hyperpolarization (Fig. 2, C and D), which was prevented by amiloride (Fig. 2, E and F). Altogether these results indicate that activation of PAR2 induces a marked increase in electroneutral Na+ transport and a small stimulation of electrogenic transport in the CCD.

PAR2 and Sodium Homeostasis—To evaluate in vivo the impact of the PAR2-mediated control of Na+ transport in CCD, we compared the renal handling of Na+ in WT and PAR2−/− mice. The two mouse lines displayed similar baseline plasma electrolyte concentrations, pH, hematocrit, and
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Biological parameters at baseline in wild type and PAR2−/− mice

|                  | Wild type | PAR2−/− |
|------------------|-----------|---------|
| **Blood/plasma** |           |         |
| Na⁺ (mEq/L)      | 147.4 ± 0.4 (6) | 149.2 ± 0.5 (5) |
| K⁺ (mEq/L)       | 4.6 ± 0.1 (7) | 4.5 ± 0.2 (6) |
| Ca²⁺ (mEq/L)     | 1.30 ± 0.02 (6) | 1.31 ± 0.03 (5) |
| HCO₃⁻ (mEq/L)    | 23.7 ± 1.8 (6) | 25.9 ± 2.0 (5) |
| PCO₂ (mmHg)      | 45.3 ± 4.6 (6) | 50.4 ± 5.0 (5) |
| pH               | 32.7 ± 0.6 (6) | 31.2 ± 0.7 (5) |
| Anion gap (mEq)  | 7.34 ± 0.03 (6) | 7.33 ± 0.02 (5) |
| Systolic blood pressure (mmHg) | 105.9 ± 1.5 (6) | 103.1 ± 2.1 (5) |
| Hematocrit (%)   | 42.2 ± 1.2 (6) | 41.6 ± 1.1 (5) |
| **Urine**        |           |         |
| Na⁺/Cr (mmol/mmol) | 33.9 ± 1.5 (6) | 34.5 ± 4.7 (6) |
| K⁺/Cr (mmol/mmol) | 21.5 ± 1.7 (6) | 21.4 ± 0.5 (6) |
| Ca²⁺/Cr (mmol/mmol) | 0.38 ± 0.02 (12) | 0.39 ± 0.02 (11) |
| Mg²⁺/Cr (mmol/mmol) | 5.2 ± 0.4 (12) | 4.9 ± 0.3 (11) |
| NH₄⁺/Cr (mmol/mmol) | 35.1 ± 0.4 (6) | 38.6 ± 4.2 (6) |
| pH               | 6.25 ± 0.07 (8) | 6.41 ± 0.11 (6) |
| Aldosterone/Creat (µg/mmol) | 4.8 ± 0.6 (17) | 3.9 ± 0.7 (12) |

TABLE 1

Blood pressure and similar rates of urinary excretion of electrolytes and aldosterone (Table 1). This apparently normal handling of Na⁺ results from mutually compensating functional changes in ASDN of PAR2−/− mice. They displayed reduced electroneutral and increased electrogenic Na⁺ reabsorption in ASDN, as attested by their decreased and increased natriuretic response to HCTZ and amiloride, respectively (Fig. 3A). In contrast, PAR2−/− mice displayed normal function of their thick ascending limb of Henle’s loop because furosemide induced the same changes in natriuresis and calcium excretion in WT and PAR2−/− mice (Fig. 3A and B). Because acute thiazide stimulates Ca²⁺ reabsorption in the DCT (13) but not in CCD (14), the fact that WT and PAR2−/− mice displayed the same changes in Ca²⁺ excretion in response to HCTZ (Fig. 3B) suggests that reduced electroneutral Na⁺ reabsorption in PAR2−/− mice does not originate from DCTs.

To emphasize these differences in Na⁺ handling between WT and PAR2−/− mice, we boosted Na⁺ reabsorption in ASDN by feeding the mice an Na⁺-depleted diet, a treatment known to induce electroneutral reabsorption of NaCl in WT mice (7). The decrease in urinary Na⁺ excretion observed during dietary Na⁺ restriction was significantly blunted in PAR2−/− mice (Fig. 4A). In addition PAR2−/− mice developed hypertonia, whereas WT mice did not (Fig. 4B), demonstrating their inability to maintain their volemia.

In vitro microperfusion showed that Na⁺ reabsorption in CCDs from Na⁺-depleted mice was markedly blunted in

FIGURE 2. Effect of PAR2 activation on sodium transport in the CCD. A, sodium reabsorption flux ($J_{Na^+}$, in picoequivalent of Na⁺/mm/min) was measured in normal rat CCDs under basal condition (B) or after the addition of 10 nM trypsin (Try) to the bath. CCDs were pretreated or not with 10 μM amiloride (Am) or 100 μM HCTZ before trypsin addition. Data are means ± S.E. from n CCDs. **, p < 0.005 as compared with trypsin condition by variance analysis. B, sodium reabsorption flux ($J_{Na^+}$, in picoequivalent of Na⁺/mm/min) measured in CCDs from WT and PAR2−/− mice under basal conditions (B) or after the addition of 10 nM trypsin (Try). Data are means ± S.E. from n CCDs. **, p < 0.005 as compared with basal conditions by Student’s t test. C, representative traces showing the variations of PDte in a rat CCD after sham change of bath solution (Sham, top) or change to a solution containing 10 nM trypsin (Try, bottom). Viability of CCDs at the end of the experimental period was attested by the hyperpolarizing effect of 0.2 nM AVP. D, mean variation of PDte (ΔPDte) after sham change (Control) or the addition of trypsin (Try). ΔPDte values were calculated between the change in bath solution and the peak depolarization (2−5 min), the peak hyperpolarization (6−10 min), and 4−8 min after peak depolarization. Values are means ± S.E. from n CCDs. ***, p < 0.001; **, p < 0.005; *p < 0.05 as compared with control by Student’s t test. E and F, same as in C and D in the presence of 10 μM amiloride in the perfusate.

FIGURE 3. Effect of diuretics on sodium and calcium excretion in wild type and PAR2−/− mice. A and B, urinary excretion of Na⁺ ($U_{Na^+}$) and Ca²⁺ ($U_{Ca^{2+}}$) before and after a single administration of diuretics, at time 0, to PAR2−/− (full lines) and WT mice (dotted lines). The diuretics were administered as: HCTZ (50 mg/kg of body weight, intraperitoneally), amiloride (Amilo, 5 mg/kg of body weight, intraperitoneally), and furosemide (Furo, 10 mg/kg of body weight, intraperitoneally). Values (in mmol/mmol creatinine ($U_{Creat}$)) are means ± S.E. from 8−12 WT and PAR2−/− mice. *, p < 0.005 versus WT by Student’s t test.
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![Graphs and images](https://example.com/graphs)

**FIGURE 4. Role of PAR2 in the renal handling of sodium.** A–D, daily urinary excretion of Na\(^+\) (U\(_{\text{Na}}\)) and Ca\(^{2+}\) (U\(_{\text{Ca}}\)) (A and D) in mmol of Na\(^+\) or Ca\(^{2+}\)/mmol of creatinine (U\(_{\text{creat}}\)) and systolic blood pressure (B) in PAR2\(^{-/-}\) (solid line) and WT mice (dashed line) fed either a control diet (C) or an Na\(^+\)-depleted diet (LN) containing 3 and 0.11 g of Na\(^+\)/kg, respectively. Values are means ± S.E. from 5 WT and 9 PAR2\(^{-/-}\) mice. ***, p < 0.001; **, p < 0.005 and *, p < 0.05 as compared with WT by Student’s t test. C, sodium reabsorption flux (J\(_{\text{Na}}\)) in picoequivalent of Na\(^+\)/mm/min was determined under control conditions (C) or in the presence of 10 \(\mu\)M amiloride (Am) in CCDs from WT and PAR2\(^{-/-}\) fed the Na\(^+\)-depleted diet for 2 weeks. Values are means ± S.E. from five WT and four PAR2\(^{-/-}\) mice. ***, p < 0.001 and **, p < 0.005 by variance analysis.

**FIGURE 5. Role of ERK in PAR2 signaling.** A, representative immunoblot and mean phospho-ERK/ERK ratio in rat CCDs incubated for 10 min at 37 °C under basal conditions (B) or in the presence of 40 \(\mu\)M AP. Values are means ± S.E. from five experiments. *, p < 0.05 by Student’s t test. Ph-ERK, phospho-ERK. B, the effect of 10 \(\mu\)M U0126 on trypsin (Try)-stimulated sodium reabsorption flux (J\(_{\text{Na}}\)) in rat CCDs. Data are means ± S.E. from five experiments. *, p < 0.05 by Student’s t test. C, mean APDM after the addition of furoylated PAR2 agonist peptide (F-AP, 10 \(\mu\)M) in rat CCDs pretreated (solid line) or not (stippled line) with U0126 (10 \(\mu\)M) for 20–30 min. Values are means ± S.E. from 5 CCDs. ***, p < 0.005 as compared with F-AP by Student’s t test. D, Na,K-ATPase activity in rat CCDs pretreated or not with 10 \(\mu\)M U0126 for 45 min at 30 °C before incubation for 10 min at 37 °C in the absence (B) or presence of 40 \(\mu\)M AP. Values are means ± S.E. from four experiments. ***, p < 0.001 as compared with B Student’s t test.

PAR2\(^{-/-}\) mice, mainly due to the absence of its electroneutral, thiazide-sensitive moiety (Fig. 4C). Thus, PAR2 is not only able to acutely activate electroneutral Na\(^+\) reabsorption in CCD, but it is also required for the induction of this transport pathway in response to Na\(^+\) depletion. Na\(^+\) depletion also increased the abundance of PAR2 mRNAs in CCDs from WT mice (in arbitrary units ± S.E.; control, 3.9 ± 0.3, n = 5; Na\(^+\)-depleted, 7.1 ± 0.4, n = 5; p < 0.001). Because proteolytic activation of PAR2 is followed by its rapid endocytosis, degradation, and de novo synthesis (15), increased abundance of PAR2 mRNAs is considered as an index of earlier activation of the receptor.

The abolition of electroneutral Na\(^+\) reabsorption in CCDs from Na\(^+\)-depleted PAR2\(^{-/-}\) mice is consistent with their inability to maintain volemia. However, because we studied a mouse model with whole body invalidation of PAR2 gene, one might question the contribution of other nephron segments and other organs to the observed differences in Na\(^+\) handling and blood pressure. The similarity of renal calcium handling in WT and PAR2\(^{-/-}\) mice in response to Na\(^+\) depletion (Fig. 4D) suggests that nephron segments located upstream of the CNT participate in PAR2 action to a limited degree, if at all. Conversely, the CNT is likely involved in these effects because it expresses PAR2 (data not shown) and displays the same Na\(^+\) transport pathways as CCD. Blood pressure is also determined by the vascular tone, and PAR2 is expressed at high level in blood vessels. However, the hypotension observed in Na\(^+\)-depleted PAR2\(^{-/-}\) mice cannot be accounted for by a vascular defect because activation of vascular PAR2 induces vasodilatation (16).

**Role of ERK in PAR2-activated Na\(^+\) Reabsorption.—**Because ERK is activated by PAR2 in many cells, including kidney epithelial cells (6), and controls Na\(^+\) transporters in the ASDN (17), we next investigated its role in PAR2 signaling in CCDs. In rat CCD, activation of PAR2 increased the phosphorylation of ERK over 2-fold (Fig. 5A). Inhibition of ERK phosphorylation with U0126 (18) reduced slightly but significantly trypsin-activated Na\(^+\) reabsorption (Fig. 5B); this inhibition was of the same magnitude as that induced by amiloride (Fig. 2A). This suggests that ERK phosphorylation is necessary for the stimulation of the electrogenic but not the electroneutral Na\(^+\) reabsorption. Accordingly, U0126 inhibited PAR2-dependent hyperpolarization (Fig. 5C). As in the thick ascending limb of Henle’s loop (6), activation of PAR2 increased the activity of Na,K-ATPase, which energizes electrogenic Na\(^+\) reabsorption, and this effect was abolished by U0126 (Fig. 5D). The ~2-fold increase in Na,K-ATPase activity appears disproportionate with the limited increase in electrogenic Na\(^+\) transport. This apparent discrepancy likely stems from the fact that phosphorylation of ERK reduces ENaC activity (19).

**PAR2 and Potassium Homeostasis.—**Phosphorylation of ERK is associated with inhibition of the K\(^+\)-secreting channels (ROMK) in the CNT/CCD (20). We therefore evaluated whether PAR2 might also inhibit K\(^+\) secretion. Because in vitro microperfused CCDs display neither Na\(^+\) reabsorption nor K\(^+\)
secretion under basal condition, we first induced both transporters with vasopressin (AVP) (22). These experiments were performed in the absence of bicarbonate, which suppresses electroneutral sodium reabsorption. Tertiapin Q (TPQ, 200 nM) was added to the luminal side, whereas AVP (200 nM) and trypsin (Try, 10 μM) were added to the basolateral side. CCDs were successively treated with or without tertiapin Q (5 min) and trypsin (20 min) before the addition of AVP. Data are means ± S.E. from 4–5 CCDs. *, p < 0.05 (basal (B) versus trypsin); §, p < 0.01 (AVP versus AVP + tertiapin Q (AVP + TPQ)) by variance analysis. B, daily urinary excretion of K⁺ (UJKLMNOP delineated) in PAR2−/− (solid line) and WT mice (dashed line) fed either a control diet (C) or a K⁺-depleted diet (LK) containing 6 and <0.1 g K⁺/kg, respectively. Values are means ± S.E. from six mice. **, p < 0.005 as compared with WT by Student’s t test. C, plasma K⁺ concentration (in mM) in PAR2−/− (solid bars) and WT mice (open bars) fed either a control diet (C) or a K⁺-depleted diet (LK) for 2 days. Values are means ± S.E. from six mice. *, p < 0.05 and **, p < 0.005 by variance analysis.

FIGURE 6. Role of PAR2 on the renal transport of potassium. A, Na⁺ and K⁺ fluxes (JNa⁺ and JK⁺, respectively, in picoequivalent of Na⁺ or K⁺/mm/min) in microperfused rat CCDs. Experiments were performed in the absence of bicarbonate, which suppresses electroneutral sodium reabsorption. Tertiapin Q (TPQ, 200 nM) was added to the luminal side, whereas AVP (200 nM) and trypsin (Try, 10 μM) were added to the basolateral side. CCDs were successively treated with or without tertiapin Q (5 min) and trypsin (20 min) before the addition of AVP. Data are means ± S.E. from 4–5 CCDs. *, p < 0.05 (basal (B) versus trypsin); §, p < 0.01 (AVP versus AVP + tertiapin Q (AVP + TPQ)) by variance analysis. B, daily urinary excretion of K⁺ (UJKLMNOP delineated) in PAR2−/− (solid line) and WT mice (dashed line) fed either a control diet (C) or a K⁺-depleted diet (LK) containing 6 and <0.1 g K⁺/kg, respectively. Values are means ± S.E. from six mice. **, p < 0.005 as compared with WT by Student’s t test. C, plasma K⁺ concentration (in mM) in PAR2−/− (solid bars) and WT mice (open bars) fed either a control diet (C) or a K⁺-depleted diet (LK) for 2 days. Values are means ± S.E. from six mice. *, p < 0.05 and **, p < 0.005 by variance analysis.

To evaluate the in vivo impact of the effect of PAR2 on K⁺ secretion in CCDs, we compared renal handling of K⁺ by WT and PAR2−/− mice in response to dietary K⁺ restriction. PAR2−/− mice showed an impaired ability to reduce their urinary K⁺ excretion when fed a K⁺-depleted diet (Fig. 6B) and, accordingly, their plasma K⁺ concentration was significantly decreased 48 h after the diet onset (Fig. 6C). Within 2 days of K⁺ restriction, the abundance of PAR2 mRNAs increased in the CCDs of K⁺-depleted WT mice (in arbitrary units ± S.E.; control, 0.70 ± 0.04, n = 5; K⁺-depleted, 1.94 ± 0.07, n = 5; p < 0.001), indicating increased activation of PAR2.

The defect in K⁺ conservation observed in K⁺-depleted PAR2−/− mice is consistent with PAR2-mediated inhibition of ROMK, but here again one cannot exclude the contribution of other structures. Adaptation to K⁺ depletion involves increased reabsorption of K⁺ in the colon and K⁺ conservation by the CNT/collecting duct through inhibition of ROMK-mediated K⁺ secretion and stimulation of H,K-ATPase-mediated K⁺ reabsorption (24). Because activation of PAR2 stimulates K⁺ secretion in the colon (25), the hypokalemia observed in K⁺-depleted PAR2−/− mice (Fig. 5C) cannot be attributed to a colon defect. Whether PAR2 also controls K⁺ homeostasis through activation of H,K-ATPase in the collecting duct is an interesting hypothesis that remains to be investigated.

DISCUSSION

Present data demonstrate the expression of PAR2 at the basolateral pole of principal and intercalated cells of the renal collecting duct. Activation of PAR2 in collecting duct inhibits K⁺ secretion in principal cells via an ERK-dependent pathway and stimulates electroneutral reabsorption of NaCl independently of ERK in intercalated cells. These combined actions of PAR2 make it possible to increase the reabsorption of Na⁺, whereas preventing K⁺ secretion in collecting ducts. Results also demonstrate the involvement of PAR2 in the control of blood pressure and plasma potassium concentration. Finally, they bring the first evidence for the role of the electroneutral NaCl reabsorption pathway recently discovered in distal nephron intercalated cells in the maintenance of blood pressure.

Control of Na⁺ and K⁺ transport in the distal nephron is mainly assumed by aldosterone. Aldosterone increases Na⁺ reabsorption through stimulation of the thiazide-sensitive NaCl co-transporter in the DCT and of ENaC in the CNT/CCD and increases potassium secretion secondarily to the activation of ENaC. Because adrenal secretion of aldosterone is stimulated in response to either hypovolemia or hyperkalemia, the simultaneous stimulation of Na⁺ reabsorption and K⁺ secretion appears as a physiological paradox. In response to hypovolemia, kidneys need to increase Na⁺ reabsorption to restore extracellular volume without increasing K⁺ secretion to avoid hypokalemia, and in response to hyperkalemia, they need to increase K⁺ secretion without altering Na⁺ reabsorption. Solving this paradox supposes that the primary effects of aldosterone on Na⁺ and K⁺ transport are modulated by cofactors differentially activated during hypovolemia and hyperkalemia. Angiotensin 2 (AII), which is produced during hypovolemia but not hyperkalemia, was recently shown to be such a modulator of aldosterone one action through its effects on the with-no-lysine (WNK) kinase 4, a molecular switch enabling the ASDN to reabsorb Na⁺ or to secrete K⁺ (26). All activates NaCl co-transporter in DCT (27) and ENaC (28, 29) and pendrin (30, 31) in CNT/CCD and inhibits ROMK (32, 33). Our present findings show that activation of PAR2 globally mimics the actions of AII, but that its effects are likely restricted to the CNT/CCD. PAR2 is there-
Proteinase-activated Receptor 2 Controls Renal Ion Handling

One of the novel Nedd4-binding proteins identified by this strategy is the protease-activated receptor 2 (PAR2), which is a G protein-coupled receptor that is activated by the proteases trypsin, kallikrein, and cathepsin G (3). In the kidney, PAR2 is expressed in cortical collecting duct (CCD) principal cells (37). Thus, PAR2-mediated stimulation of NaCl reabsorption pathway in the maintenance of blood pressure. Because blood pressure is not determined by the balance of Na$^+$ but by that of NaCl (34–36), involvement of PAR2 and the thiazide-sensitive NaCl reabsorption pathway in the maintenance of blood pressure appears well adapted.

The results show that PAR2 is also involved in the regulation of Na$^+$ and K$^+$ homeostasis in a context of nonstimulation of the renin/angiotensin/aldosterone system such as K$^+$ depletion. This duality of physiological context in which PAR2 is involved likely stems from the fact that unlike most membrane receptors, which are activated by specific ligands (e.g. AII receptors), PAR2 can be activated by different protease/inhibitor complexes under different conditions. Identifying the proteases/inhibitors responsible for PAR2 stimulation in CCD under different conditions is a challenging prerequisite to explore further this new pathway involved in body homeostasis. At first glance, involvement of PAR2 in kidney adaptation to K$^+$ depletion may seem inappropriate because PAR2-mediated inhibition of ROMK is accompanied with a stimulation of electroneutral Na$^+$ reabsorption. However, it is worth mentioning that K$^+$ restriction reduces aldosterone secretion, which in turn decreases ENaC expression and Na$^+$ reabsorption in CNT/CCD principal cells (37). Thus, PAR2-mediated stimulation of Na$^+$ reabsorption by ICs may be needed precisely to compensate for ENaC down-regulation.

In summary, we identified a new role of proteases in the control of electrolyte homeostasis, blood pressure, and plasma K$^+$ concentration through their capacity to activate PAR2 in the late distal nephron. Given its role in inflammation, PAR2 is considered as a potential therapeutic target; one should keep in mind that PAR2 antagonists may induce deleterious hypokalemia as a side effect. We also identified PAR2 as a membrane receptor able to modulate sodium/potassium exchange in the distal nephron independently of the aldosterone status. Finally, we provide evidence for the role of NaCl reabsorption in distal nephron intercalated cells in the maintenance of volemia and blood pressure.

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